



PHD

Tumour necrosis factor-alpha regulation in human alveolar macrophages: mechanisms in health and acute lung injury

Armstrong, Lynne

Award date:
1995

Awarding institution:
University of Bath

[Link to publication](#)

Alternative formats

If you require this document in an alternative format, please contact:
openaccess@bath.ac.uk

Copyright of this thesis rests with the author. Access is subject to the above licence, if given. If no licence is specified above, original content in this thesis is licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC-ND 4.0) Licence (<https://creativecommons.org/licenses/by-nc-nd/4.0/>). Any third-party copyright material present remains the property of its respective owner(s) and is licensed under its existing terms.

Take down policy

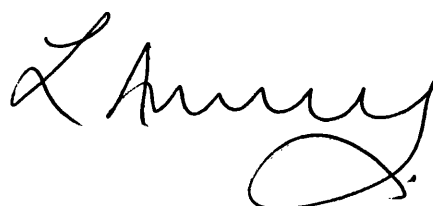
If you consider content within Bath's Research Portal to be in breach of UK law, please contact: openaccess@bath.ac.uk with the details. Your claim will be investigated and, where appropriate, the item will be removed from public view as soon as possible.

**TUMOUR NECROSIS FACTOR-ALPHA
REGULATION IN HUMAN ALVEOLAR
MACROPHAGES:
MECHANISMS IN HEALTH AND ACUTE LUNG
INJURY**

**Submitted by Lynne Armstrong
for the degree of PhD
of the University of Bath
1995**

COPYRIGHT

Attention is drawn to the fact that copyright of this thesis rests with its author. This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the prior written consent of the author.

A handwritten signature in black ink, appearing to read 'L. Armstrong', with a large, stylized loop at the end.

UMI Number: U543191

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U543191

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

UNIVERSITY OF ALABAMA	
LIBRARY	
23	23 AUG 1996
Ph.D	
5105456	

ABSTRACT

This thesis describes the regulation of alveolar macrophage (AM) and peripheral blood monocyte (PBM) derived tumour necrosis factor (TNF- α) by the cytokine interleukin-10 (IL-10) and the p-55 soluble TNF receptor (TNF-R). The relationship between TNF- α , IL-10 and TNF-R was explored first in AMs and PBMs from normal subjects and then compared to cells derived from patients with the inflammatory lung diseases sarcoidosis, cryptogenic fibrosing alveolitis (CFA) and adult respiratory distress syndrome (ARDS). The distribution of three distinct AM phenotypes (accessory, suppressor and tissue AMs) and their ability to produce TNF- α protein was also assessed in these subjects.

AMs and PBMs were isolated by bronchoalveolar lavage (BAL) and venepuncture respectively. Cells were cultured for twenty four hours to permit protein determination by enzyme-linked immunosorbent assay (ELISA), or two to four hours when determining messenger RNA (mRNA) expression. mRNA was extracted and measured by northern analysis. The bacterial cell wall component lipopolysaccharide (LPS) was added to cultures as a non-specific inflammatory stimulus, with appropriate controls.

The major findings of this thesis were as follows:

- (1). IL-10 reduced TNF- α production by AMs and PBMs in normal subjects, and this inhibition was due at least in part to suppression of TNF mRNA expression. The activity of IL-10 in this respect was not dependent upon *de novo* protein synthesis, nor did IL-10 enhance the rate of mRNA degradation.
- (2). TNF- α was enhanced in the plasma and bronchoalveolar lavage fluid (BALF) of sarcoidosis subjects when compared to normal subjects, but the AM was not the source any enhanced production. TNF-R was enhanced in the plasma and BALF and was shed by AMs and PBMs *in vitro*. IL-10 was not enhanced, but there was increased potency of IL-10 inhibition of TNF- α in AMs derived from sarcoidosis subjects.
- (3). TNF- α production by AMs derived from subjects with CFA was increased in response to LPS when compared to normal subjects. TNF-R was also increased, and the

AMs and PBMs had enhanced capacity to produce IL-10. The potency of IL-10 inhibition of TNF- α was one hundred-fold stronger than that observed in normal AMs.

(4). TNF- α production by AMs derived from subjects with ARDS was enhanced when compared to subjects with pre-disposing risk factors (at risk subjects). TNF-R was elevated in the plasma and BALF of both groups but was undetectable in the supernatants of AM and PBM cultures. BALF IL-10 was significantly elevated in the ARDS group, indicating that IL-10 may be protective in acute lung injury, and the AM may be a major source.

(5). Distribution of AM phenotypes was altered in sarcoidosis and CFA BAL in favour of accessory and suppressor AMs. They were not altered in ARDS. The suppressor population were able to produce enhanced TNF- α in culture compared to accessory and tissue AMs. Accessory AMs were unable to produce TNF- α in culture.

ACKNOWLEDGEMENTS

I would first of all like to express heartfelt appreciation to my supervisor, Dr Ann Millar for her unwavering support, both scientific and emotional, during my years as a PhD student. I am also grateful to my external supervisor Dr Len Poulter for his advice, and for providing the antibodies for the phenotypic analyses. Thanks also to Dr Sue Stephens of Celltech for provision of the TNF- α ELISA reagents.

I would also like to thank the staff and postgraduate students in the School of Pharmacology, University of Bath, especially Dr Zarin Brown and Dr Nicola Jordan, whose practical advice was invaluable. I am also extremely grateful to Mrs Jane Leithead for her technical assistance and expertise, as well as being a good listener. Many thanks also to Rachel Robson, Andrew Bourne and Mary Thompson for being supportive colleagues during my period at Bath.

Finally, I am extremely grateful to my father Norman and my sister Julie for supporting me during my student years and I am especially thankful to my husband Dr Jason Mansell for his advice, loving support and encouragement.

LIST OF ABBREVIATIONS

AM	alveolar macrophage
APC	antigen presenting cell
ARDS	adult respiratory distress syndrome
BALF	bronchoalveolar lavage fluid
CD	cluster of differentiation
CFA	cryptogenic fibrosing alveolitis
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
HLA	human leucocyte antigen
IFN- γ	interferon-gamma
IL-	interleukin-
ITU	intensive therapy unit
LPS	lipopolysaccharide
MCP-1	monocyte chemotactic protein-1
MDM	monocyte-derived macrophage
MHC	major histocompatibility complex
MIP-1 α	macrophage inflammatory protein-1-alpha
mRNA	messenger ribonucleic acid
PBM	peripheral blood monocyte
PBMC	peripheral blood mononuclear cell
PMA	phorbol myristate acetate
PMN	polymorphonuclear neutrophil
Th	T helper
TNF- α	tumour necrosis factor-alpha
TNF-R	tumour necrosis factor receptor

CONTENTS

1.	Introduction	1
1.1	Pulmonary inflammation	1
1.2	Lung immunology	3
1.2.1	Innate immunity	3
1.2.2	Acquired immunity	3
1.2.3	T lymphocytes	4
1.2.4	Polymorphonuclear neutrophils	5
1.2.5	Alveolar macrophages	6
1.3	Cytokines and pulmonary inflammation	9
1.3.1	Tumour necrosis factor	9
1.3.2	Interleukin-1- β	12
1.3.3	The Chemokines	12
1.3.4	Interleukin-10	13
1.3.5	Interleukin-4 and interleukin-13	15
1.4	Sarcoidosis	15
1.4.1	Aetiology	16
1.4.2	Pathology	17
1.5	Cryptogenic fibrosing alveolitis	19
1.5.1	Aetiology	20
1.5.2	Pathology	21
1.6	Adult respiratory distress syndrome	22
1.6.1	Aetiology	22
1.6.2	Pathology	23
1.7	Aims of the study	24

2.	Materials and methods	26
2.1	Bronchoalveolar lavage	26
2.2	Alveolar macrophage culture	26
2.2.1	Preparation of AMs from ITU patients	27
2.2.2	Preparation of AMs for sub-population analysis	27
2.3	Peripheral blood monocyte culture	28
2.3.1	Twenty-four hour monocyte culture	29
2.3.2	Monocyte-derived macrophage culture	29
2.4	Culture of cell lines	29
2.4.1	U937 cells	29
2.4.2	THP-1 cells	30
2.5	Measurement of protein by enzyme-linked immunosorbent assay	30
2.5.1	TNF- α	30
2.5.2	Interleukin-10	31
2.5.3	p-55 TNF receptor (TNF-R)	31
2.6	Detection of protein by immunohistochemistry	34
2.6.1	Cytospins	34
2.6.2	TNF- α immunostaining	34
2.6.3	IL-10 immunostaining	36
2.6.4	AM phenotype analysis	36
2.6.5	CD68 immunostaining	37
2.7	Northern analysis	37
2.7.1	Cell culture	37
2.7.2	RNA extraction	37
2.7.3	Gel electrophoresis of RNA samples	40
2.7.4	Probing of nylon membranes	41
2.7.5	Densitometry	41

2.8	Statistical analysis	42
3.	Tumour necrosis factor-α regulation by interleukin-10 in normal subjects	43
3.1	Introduction	43
3.2	Experimental design	45
3.3	Results	47
3.3.1	IL-10 suppresses the production of extracellular TNF- α from LPS-treated AMs and PBMs in a dose dependent manner	47
3.3.2	IL-10 inhibits the expression of TNF- α mRNA from LPS-challenged AMs	47
3.3.3	The effect of IL-10 pre- and post-treatment of TNF- α mRNA levels from LPS-treated PBMs	47
3.3.4	IL-10 inhibits the expression of TNF mRNA from LPS-challenged PBMs	51
3.3.5	IL-10 induced suppression of TNF- α is not dependent on <i>de novo</i> protein synthesis	51
3.3.6	The effect of IL-10 addition on the rate of TNF mRNA decay	51
3.4	Discussion	56
4.	TNF-α regulation in sarcoidosis	59
4.1	Introduction	59
4.2	Experimental design	62
4.3	Results	63
4.3.1	TNF- α in plasma and BALF	63
4.3.2	TNF- α release by AMs and PBMs spontaneously and in response to LPS	63
4.3.3	Modification of TNF- α release by IL-10	63
4.3.4	IL-10 levels in plasma and BALF	67
4.3.5	IL-10 release by AMs and PBMs spontaneously and in response to LPS	67
4.3.6	TNF-R levels in plasma and BALF	67
4.3.7	TNF-R release by AMs and PBMs spontaneously and in response to LPS	71

4.3.8	Modification of TNF-R release by IL-10	71
4.4	Discussion	74
5.	TNF-α regulation in cryptogenic fibrosing alveolitis	78
5.1	Introduction	78
5.2	Experimental design	81
5.3	Results	82
5.3.1	TNF- α in plasma and BALF of CFA subjects	82
5.3.2	Spontaneous and LPS-induced TNF- α release by AMs and PBMs from CFA subjects	82
5.3.3	TNF- α inhibition by IL-10 in AMs and PBMs from CFA subjects	82
5.3.4	IL-10 in plasma and BALF of CFA subjects	86
5.3.5	Spontaneous and LPS-induced IL-10 production by AMs and PBMs from CFA subjects	86
5.3.6	TNF-R in the plasma and BALF of CFA subjects	86
5.3.7	Spontaneous and LPS-induced TNF-R release by AMs and PBMs from CFA subjects	86
5.3.8	The effect of IL-10 on TNF-R release by AMs and PBMs from CFA subjects	91
5.4	Discussion	93
6.	TNF-α regulation in adult respiratory distress syndrome	97
6.1	Introduction	97
6.2	Experimental design	100
6.3	Results	102
6.3.1	TNF- α in the plasma and BALF of ARDS and at risk subjects	102
6.3.2	Spontaneous and LPS-induced TNF- α release from AMs and PBMs	102
6.3.3	Inhibition of LPS-induced TNF- α production by IL-10	102

6.3.4	IL-10 in the plasma and BALF of ARDS and at risk subjects	106
6.3.5	Spontaneous and LPS-induced IL-10 release by AMs and PBMs	106
6.3.6	TNF-R in the plasma and BALF of ARDS and at risk subjects	109
6.3.7	TNF-R production by AMs and PBMs from ARDS and at risk subjects	109
6.4	Discussion	111
7.	Alveolar macrophage populations in inflammatory lung disease	115
7.1	Introduction	115
7.2	Experimental design	119
7.2.1	Phenotypic distribution in normal BAL and the BAL of subjects with lung disease. The ability of sub-populations to produce TNF- α	119
7.2.2	Induction of RFD1 and RFD7 expression in MDMs, THP-1 and U937 cells	119
7.3	Results	120
7.3.1	Distribution of RFD antigens in normal BAL	120
7.3.2	Distribution of RFD antigens in sarcoidosis BAL	120
7.3.3	Distribution of RFD antigens in CFA BAL	120
7.3.4	Distribution of RFD antigens in the BAL of subjects at risk of ARDS	124
7.3.5	Distribution of RFD antigens in the BAL of subjects with ARDS	124
7.3.6	Intra-cellular TNF- α production and protein release by sub-populations from normal BALF	124
7.3.7	Induction of RFD1 and RFD7 in cultured unstimulated monocyte-derived macrophages	126
7.3.8	The effect of LPS and PMA treatment on RFD antigen expression in MDMs	131
7.3.9	The effect of IFN-g on RFD expression on MDMs	131
7.3.10	The effect of dex on RFD expression on MDMs	131
7.3.11	The effect of IFN- γ and dex co-culture on RFD expression on MDMs	135
7.3.12	RFD and CD68 expression on undifferentiated U937 and THP-1 cells in culture	135
7.3.13	Induction of RFD antigens on THP-1 cells in response to IL-1- α , retinoic acid (RA) and dibutyryl cAMP	135

7.3.14	Induction of RFD antigens in U937 by PMA and vitamin D ₃	138
7.4	Discussion	140
8	General discussion	144
9	Bibliography	151

List of Figures:

1.1	The alveolus	1
1.2	Extravasation of PMNs	5
1.3	Macrophage maturation	6
1.4	Proposed mechanisms of lung injury in ARDS	24
2.1	TNF- α ELISA standard curve	32
2.2	IL-10 ELISA standard curve	33
2.3	TNF-R ELISA standard curve	35
3.1	The effect of IL-10 administration on LPS-induced TNF- α release by AMs and PBMs	48
3.2	IL-10 inhibition of TNF- α expression by AMs	49
3.3	The temporal window for the efficacy of IL-10 co-administration with LPS	50
3.4	The effect of a dose range of IL-10 on TNF- α expression in PBMs	52
3.5	The requirement for <i>de novo</i> protein synthesis to mediate the effects of IL-10	53
3.6	mRNA degradation in response to IL-10 and LPS in PBMs	55
4.1	TNF- α detected in the plasma and BALF of sarcoidosis patients	64
4.2	The effect of LPS on TNF- α release by AMs and PBMs derived from patients with sarcoidosis	65
4.3	The effect of IL-10 on TNF- α release by AMs and PBMs derived from	

	patients with sarcoidosis	66
4.4	IL-10 detected in the plasma and BALF of sarcoidosis patients	68
4.5	The effect of LPS on IL-10 release by AMs and PBMs derived from patients with sarcoidosis	69
4.6	TNF-R detected in the plasma and BALF of sarcoidosis patients	70
4.7	The effect of LPS on TNF-R release by LPS in AMs and PBMs derived from sarcoidosis patients	72
4.8	The effect of IL-10 on TNF-R release by AMs and PBMs derived from patients with sarcoidosis	73
5.1	TNF- α detected in the plasma and BALF of CFA subjects	83
5.2	The effect of LPS on TNF- α release by AMs and PBMs derived from CFA subjects	84
5.3	The effect of IL-10 on TNF- α release by AMs and PBMs derived from CFA patients	85
5.4	IL-10 detected in the plasma and BALF of CFA subjects	87
5.5	The effect of LPS on IL-10 production by AMs and PBMs derived from subjects with CFA	88
5.6	TNF-R detected in the plasma and BALF of CFA subjects	89
5.7	The effect of LPS on TNF-R production by AMs and PBMs derived from CFA subjects	90
5.8	The effect of IL-10 and LPS on TNF-R release by AMs and PBMs derived from CFA subjects	92
6.1	TNF- α detection in the plasma and BALF of subjects with ARDS and at risk of ARDS	103
6.2	Spontaneous and LPS-induced TNF- α release from AMs and PBMs derived from ARDS and at risk subjects	104
6.3	The effect of IL-10 administration on LPS-induced TNF- α release from AMs and PBMs derived from ARDS and at risk subjects	105
6.4	IL-10 detected in the plasma and BALF of subjects with ARDS and at risk	

	of ARDS	107
6.5	Spontaneous and LPS-induced IL-10 release by AMs and PBMs derived from subjects with ARDS and at risk of ARDS	108
6.6	TNF-R detected in the plasma and BALF of subjects with ARDS and at risk of ARDS	110
7.1	Distribution of RFD1 and RFD7 in AMs derived from normal subjects	121
7.2	Distribution of RFD1 and RFD7 in AMs derived from subjects with sarcoidosis	122
7.3	Distribution of RFD1 and RFD7 in AMs derived from subjects with CFA	123
7.4	Distribution of RFD1 and RFD7 in AMs derived from subjects at risk of ARDS	127
7.5	Distribution of RFD1 and RFD7 in AMs derived from subjects with ARDS	128
7.6	Spontaneous and LPS-induced TNF- α production by AM sub-populations	129
7.7	Expression of RFD1 and RFD7 phenotypic markers on PBMs cultured in RPMI and 10% FCS	130
7.8	Effect of LPS and PMA on RFD1 and RFD7 expression on PBMs in culture	132
7.9	Effect of IFN- γ on RFD1 and RFD7 expression on cultured PBMs	133
7.10	Effect of dex on RFD1 and RFD7 expression on cultured PBMs	134
7.11	Expression of CD68 on unstimulated U937 and THP-1 cells	136
7.12	Effect of RA, dibutyryl cAMP and IL-10 on macrophage in THP-1 cells	137
7.13	Effect of PMA and vitamin D ₃ on macrophage differentiation in U937 cells	139
8.1	Mechanisms of activation of STATs in signalling by the cytokine receptor superfamily	145
8.2	Putative regulatory network for TNF- α AMs from normal subjects	147

List of Plates:

1.1	The alveolar macrophage	7
2.1	RFD immuno-fluorescent staining	38
2.2	CD68 immuno-fluorescent staining	39

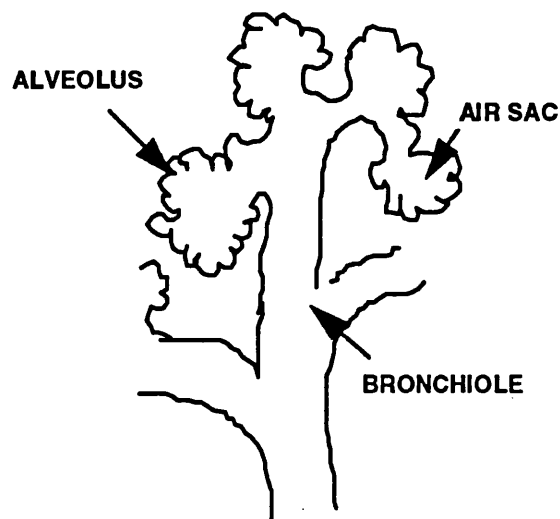
1. INTRODUCTION

The introduction to this thesis will provide an overview of pulmonary inflammation and cell/cytokine regulation in the normal lung. In addition, the mechanisms of three pulmonary inflammatory disorders; sarcoidosis, cryptogenic fibrosing alveolitis (CFA) and adult respiratory distress syndrome (ARDS) will be described. Investigation into the inflammatory processes in these diseases, with particular reference to the production of the cytokine tumour necrosis factor alpha (TNF- α) by alveolar macrophages (AMs) formed the basis for this study.

1.1 Pulmonary Inflammation

The lung is ideally suited anatomically to fulfill its role as the organ of gas exchange. As the airways branch from the trachea, through the bronchi and bronchioles, they terminate at the air sacs, which contain many convolutions, or alveoli within them. There are 10^9 pulmonary alveoli in the adult lung, providing an enormous surface area required for efficient gas exchange. This is further facilitated by the structure of the alveolar walls, which consist of flat, thin epithelial cells in immediate proximity to the pulmonary capillary network (Keele *et al.* 1982).

Figure 1.1 The Alveolus



The pulmonary epithelium represents the body's most fragile interface with the environment. The airways are exposed to a constant stream of environmental particulate matter, which may or may not be intrinsically harmful. Should microbial pathogens or antigens be retained in the airways the lung has highly efficient local mechanisms, utilising both the innate and acquired immune systems, to remove them. An inflammatory response usually ensues, which in most circumstances leads to rapid and effective clearance of particles. The fragility of the airways and the intimate association between structure and function of the lung highlights the importance of homeostatic mechanisms to maintain and protect the lung environment. It has become apparent that inflammatory responses, although representing a vital mechanism of host defence, can actually inflict lung injury itself, and in the case of an excessive response may lead to more injury than the supposed pathogen could itself alone inflict (Walters and du Bois 1995). This is particularly true in circumstances where the pathogen may be local viruses or environmental debris incapable of inflicting physical damage. Inflammation harms the host environment when mechanisms normally used to kill organisms such as parasites and bacteria are permitted to inflict local cell damage (see section 1.2.4). This can lead to loss of alveolar structure and the development of pulmonary fibrosis with the concomitant reduction in gas exchange. In the normal lung there are sophisticated mechanisms in place to distinguish between harmful pathogens and non-pathogenic environmental antigens, and so prevent unnecessary inflammation. However, there are incidences where an inappropriate inflammatory response can develop, which, if persistent, can lead to serious destruction of the pulmonary structure. Lung diseases which may be characterised by inappropriate responses include the granulomatous disorder sarcoidosis and the interstitial lung diseases cryptogenic fibrosing alveolitis (CFA) and adult respiratory distress syndrome (ARDS). All three disorders have an unknown aetiology, although ARDS is commonly associated with sepsis syndrome, and there is a consensus that they may be disorders of immune regulation.

1.2 Lung immunology

1.2.1 Innate immunity

Innate immunity is the most primitive form of immunological protection and combines barrier protection with non-memory cell-mediated protection (Stites and Terr, 1987). Since the airways are under bombardment from environmental particulate matter, barrier protection is very important. It mainly comprises of a mucus secretion which traps debris which is then removed from the airways by beating ciliated epithelium acting as a mucilliary escalator. The airways are also patrolled by phagocytic cells of the myeloid lineage of haemopoietic cells, most notably AMs of the alveolar space and polymorphonuclear neutrophils (PMNs) which enter the lungs during inflammation. When these cells encounter debris they activate complement and release acute phase proteins such as C reactive protein, which coat, or opsonise bacteria, so promoting their ingestion by phagocytes. In addition complement has an enzymatic action allowing it to lyse bacterial cell walls and host cells infected with microorganisms. AMs also have a large battery of soluble mediators, the most important of these in terms of innate immunity are TNF- α and interferon gamma (IFN- γ) (Mantovani and Dejana, 1989). Both of these cytokines are important for activation of AM killing of bacterial cells or infected host cells, and IFN- γ is particularly effective in triggering autolysis in virally infected cells (Mantovani and Dejana, 1989). The innate immune system does not work in isolation however, since components of acquired and cell-mediated immunity are activated in response to the particulate matter itself, and also in response to the mediators released by activated AMs and PMNs.

1.2.2 Acquired immunity

This response developed later in evolutionary terms than innate immunity and depends on cells of the lymphoid, rather than the myeloid lineage i.e. T and B lymphocytes (Stites and Terr, 1987). Acquired immunity is based upon immunological memory; T cells resident in lymphoid tissues such as bronchus-associated lymphoid tissue (BALT) screen antigen (see section 1.2.3), and should they encounter antigenic material they induce clonal expansion of antigen-specific B cells leading to production of large amounts of

specific antibody. Antibody binding to antigen promotes clearance by triggering ingestion by phagocytic cells through its interaction with the Fc receptor, or by attracting the attention of complement, cytotoxic T cells, AMs or natural killer cells, a type of lymphocyte capable of cell killing. Antibodies can also neutralise bacterial toxins.

1.2.3 T lymphocytes

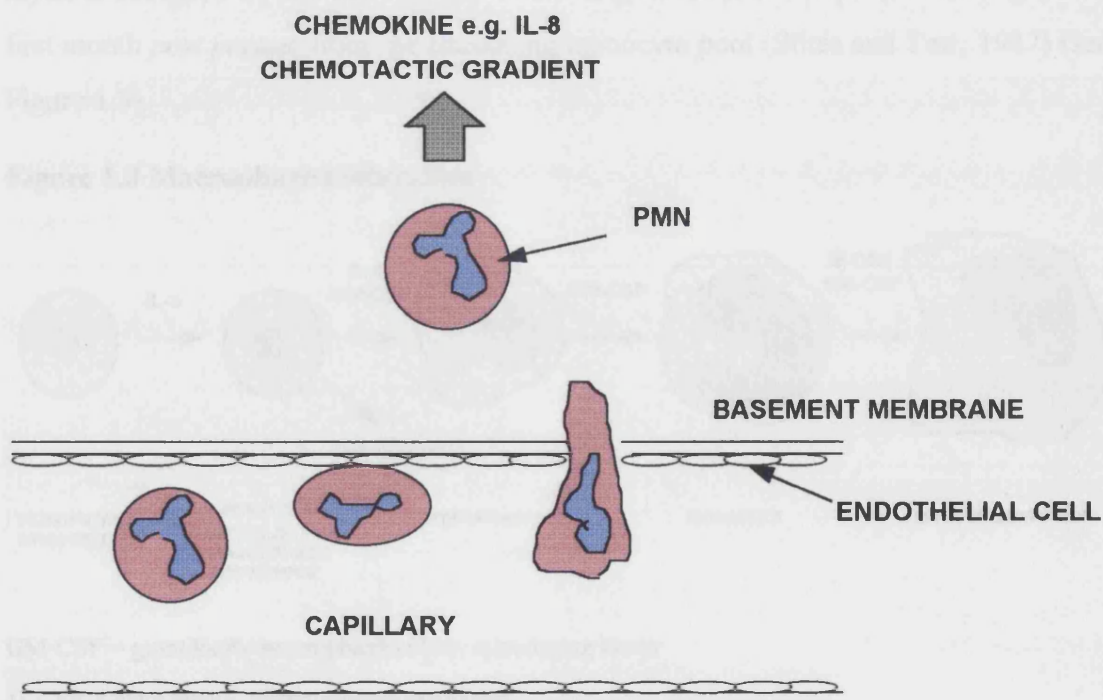
T cells are lymphocytes which have been “educated” in the thymus of the foetus. While resident in this lymphoid gland the populations of immature T cells are decimated, since cells recognising self antigens as foreign are eliminated by apoptosis, a vital process to prevent the onset of autoimmune disease (Stites and Terr, 1987). Identification of self is mediated through the expression of major histocompatibility complex (MHC) or human leucocyte antigens (HLA). Class I MHC, or HLA-A, B and C are ubiquitous, found on all somatic cells, whereas class II or HLA-DR, DQ and DP are found on all antigen presenting cells (APCs) such as macrophages, dendritic cells (DCs), peripheral blood monocytes (PBMs) and B cells (Roitt *et al.* 1989). T cells can be divided into two major subsets based on the expression of CD4 and CD8 cluster of differentiation markers. CD4, or T helper (Th) cells recognise class II MHC, whereas CD8 or T cytotoxic/suppressor (Tc/s) cells recognise class I MHC. T helper cells are so-called since they “help” or promote the establishment of an inflammatory response once they have encountered antigen processed by APCs in conjunction with class II MHC. They can be further divided into Th1 and Th2 on the basis of their cytokine profiles; Th1 cells produce IFN- γ , IL-2 and IL-3, whereas Th2 cells release IL-4, IL-5 and IL-10 (Mosmann *et al.* 1986). These distinctions were made in mouse T cells populations, however and human T cells do not appear to totally conform to this pattern (Kelso, 1995). CD8 cells, in contrast to CD4 are thought to have a role in dampening down the inflammatory response. In the normal lung the number of T cells is usually less than five per cent of the total inflammatory cells (Walters and du Bois 1995) and CD8 outnumber CD4 by approximately 2:1. This is thought to be an important mechanism for preventing inappropriate inflammatory responses. A ratio of 2:1 CD4:CD8, however, is a feature of sarcoidosis (Spiteri *et al.* 1992) and this T cell balance promotes a more vigorous

response in terms of cytokine release, B cell activation, AM activation and granuloma formation (see section 1.4).

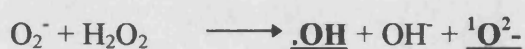
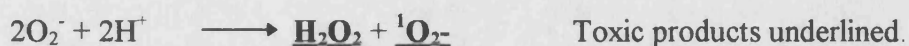
1.2.4 Polymorphonuclear neutrophils

PMNs are the dominant immune cell in the circulation, accounting for sixty to seventy per cent of circulating leucocytes. There are few in the normal lung, but during an inflammatory response PMNs are drawn in by chemokines such as interleukin-8 (IL-8) (Downey *et al.* 1993) (see section 1.3.3). The first step in extravasation of PMNs is the up-regulation of adhesion molecules L-selectin, CD18 and CD11 on the PMN and their ligands E-selectin and ICAM-1 (inter-cellular adhesion molecule-1) on vascular endothelial cells (MacNee and Selby, 1993). This up-regulation can occur in response to a range of cytokines, including IL-8, TNF- α and IL-1 (MacNee and Selby, 1993), and their interaction causes rolling and then adhesion of PMNs to the endothelium (Figure 1.2). Once stationary, the PMNs undergo morphological change, or diapedesis, allowing them to squeeze through the EC barrier, across the interstitium and into the alveolar space.

Figure 1.2 Extravasation of PMNs



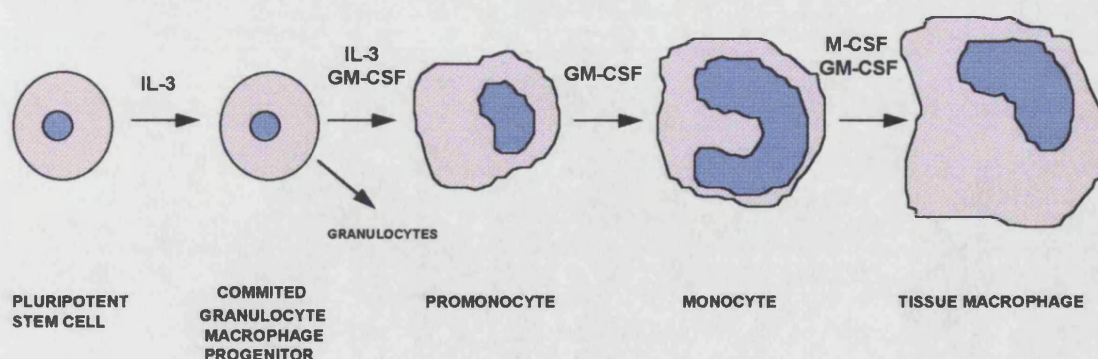
The PMN is a multi-functional cell. As part of the myeloid lineage, it is an efficient phagocyte, engulfing particulate matter and digesting it in phagolysosomes packed with proteolytic enzymes (Ogawa *et al.* 1981). Phagocytosis, and activation by C5a (complement), FMLP (bacterial cell wall component), immune complexes, opsonised microbes and cytokines can lead to PMN degranulation and the release of these enzymes (collagenase, lysozyme, peroxidase) and the respiratory burst of superoxide anions (Nathan, 1989). These can all inflict damage not only to invading organisms, but to the host tissues themselves.



1.2.5 Alveolar macrophages

Although T cells and PMNs are important effector cells in pulmonary inflammation, they are not apparent in significant numbers in the normal lung. In fact AMs account for more than ninety per cent of pulmonary inflammatory cells (see Plate 1.1). Derived from the myeloid lineage, they are not resident in the lung at birth, but populate the lung in the first month *post partum* from the circulating monocyte pool (Stites and Terr, 1987) (see Figure 1.3).

Figure 1.3 Macrophage maturation

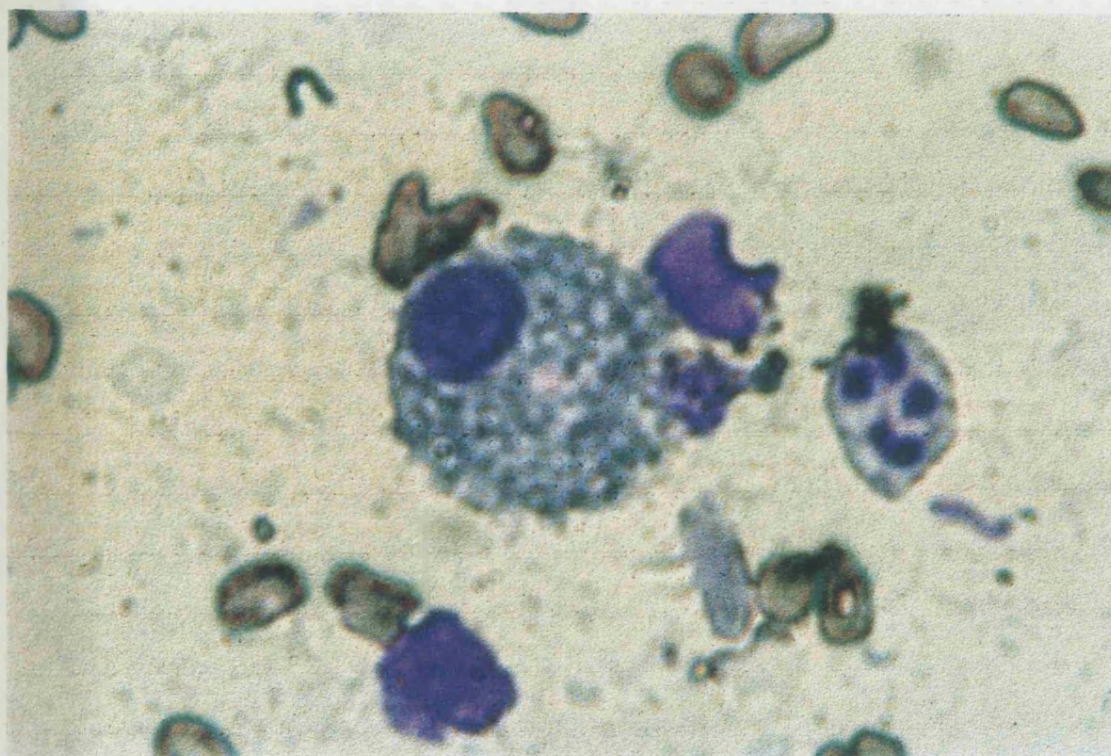


GM-CSF = granulocyte macrophage colony stimulating factor

M-CSF = macrophage colony stimulating factor

IL-3 = interleukin-3

Plate 1.1 The Alveolar Macrophage



In the adult it is thought that the majority of AMs are derived directly from PBMs (Henson and Riches, 1994) although there have been *in vitro* studies suggesting that AMs are capable of local proliferation (Nakata *et al.* 1991). It has also become apparent that AMs are not a heterogeneous population of cells, but may consist of at least three different phenotypes, separated on the basis of cell density and/or surface antigen expression (Spiteri and Poulter, 1991). These sub-populations differ in their ability to present antigen to T cells and to phagocytose debris. Such differences may be a feature of different maturation states, but it is also possible that modulation or phenotype switching in existing resident AMs may occur.

As AMs mature from PBMs they undergo alterations to their surface marker expression, increasing expression of CD68 macrophage maturation marker, and decreasing CD14 (the lipopolysaccharide receptor) expression (Stites and Terr, 1987). They are also less efficient APCs. Accessory cell function is an important property of AMs since they are the scavenger cells of the normal lung and are the first to detect foreign matter. They are capable of processing antigen and presenting it in association with class II MHC to resident T helper cells but in normal AMs this ability is much reduced. It is believed that in the normal lung dendritic cells (DCs) are responsible for most initial antigen presentation and they migrate from the interstitium to the BALT in order to elicit T cell activation. It is thought that only after activation by Th1-derived IFN- γ (which up-regulates class II MHC) are AMs capable of efficient accessory cell activity (Krakauer and Oppenheim, 1993). The requirement for priming is probably very important in the prevention of potentially hazardous inappropriate inflammatory response. Thus the major role for the resting AM is phagocytosis of debris, and it achieves this in the same manner as PMNs, although the AM is a more aggressive phagocyte. Cytokine production by AMs is usually reduced as they mature from PBMs (Kotloff *et al.* 1990; Elias *et al.* 1988), with the exception of TNF- α , which is an important cytokine for the microbicidal activity of AMs (Kelley, 1990). However, they do possess an armamentarium of over one hundred different soluble mediators. These mediators, including cytokines, prostaglandins and leukotrienes can then act on other effector cells such as T cells, ECs and PMNs in a paracrine manner and/or autocrine manner on AMs. Thus AMs have the capability to produce an array of factors involved in the initiation and maintenance of the pulmonary inflammatory response. In the normal lung it appears that there is very tight

regulation of AM activity to prevent excessive cytokine release in response to immunogens which may be intrinsically harmless. A loss of regulation could lead to a chronic inflammatory response, so it seems that the inflammatory status of the AM may be important in the development of pathological inflammatory lung disease.

1.3 Cytokines and pulmonary inflammation

Cytokines are peptide mediators of inflammation with a molecular weight of 7 - 30 kilodaltons. Their actions are similar to endocrine hormones since they can elicit responses such as cell mitosis and metabolic changes as well as modification of gene expression and protein synthesis in many cells of the body. Where they differ from hormones is their source; hormones are secreted by discrete endocrine glands and usually impinge upon cells at a distant site, via the circulation. Cytokines, by contrast, can be produced by many different cell types and usually act locally in an autocrine or paracrine fashion. They also have a short half-life. Cytokines are fundamental in orchestrating the inflammatory response of the lung and the network is usually under tight regulation to prevent inappropriate inflammation.

1.3.1 Tumour necrosis factor

TNF- α was so-called since it was initially described as a mediator of necrosis in transplantable tumours in mice (Carswell *et al.* 1975). However, it has since become apparent that TNF- α is a cytokine with a widely pleiotropic range of activity, including microbicidal activity, inflammatory effects and promotion of bone resorption (Fiers 1991). TNF- α is a 17.5 kDa polypeptide derived from a gene on the short arm of chromosome six, closely linked to the HLA locus and TNF- β . Pulmonary TNF- α can be derived from a number of sources including T cells (Fiers, 1991) and PMNs (Xing *et al.* 1993), but the majority of TNF- α in the lung is thought to be derived from the AM (Strieter *et al.* 1989a). AMs are one of the first lines of defence in the lung, being the dominant sentinel cell (Cohen and Cline, 1971) and following activation by lipopolysaccharide/endotoxin (LPS), a component of gram negative bacterial cell walls,

there is a three-fold increase in TNF- α mRNA and a hundred-fold increase in protein translation and release (Han *et al.* 1990; Beutler *et al.* 1986). This up-regulation occurs very rapidly, with mRNA increased at 1-2 hours, and the first protein release at 3-4 hours, optimal at 12-16 hours (Frei *et al.* 1993). Elevated levels of LPS are a feature of septic shock, a disease where patients have a high probability of developing acute lung injury, which may result in the adult respiratory distress syndrome (ARDS) (Fowler *et al.* 1983; Ashbaugh *et al.* 1967) and the response of AMs to LPS does suggest a possible role for TNF- α in this disease. Plasma TNF- α , likely to be derived from activated PBMs, is elevated in septic shock and ARDS, and it may correlate to severity and mortality (Cannon *et al.* 1990; Damas *et al.* 1989). TNF- α has also been found to be elevated in the bronchoalveolar lavage fluid (BALF) of patients with ARDS (Suter *et al.* 1992) and other forms of lung injury. Interest in the role of TNF- α in acute lung injury led to the development of animal models, which demonstrated that TNF- α infusion could lead to lung injury in animals (Tracey *et al.* 1987b; Tracey *et al.* 1986; Lo *et al.* 1992), and that these infusions could mimic the effects of LPS infusion models (Beutler *et al.* 1985). Further studies using anti-TNF antibodies have demonstrated protection against lethality in LPS animal models of injury including baboons (Tracey *et al.* 1987a) and mice (Beutler *et al.* 1985). More recently an animal model using mice transgenic for the TNF- α gene, (resulting in pulmonary over-expression of TNF- α) demonstrated the development of lethal interstitial pneumonitis (Piguet *et al.* 1995). The major response of the lung to TNF- α infusion are pulmonary oedema (Stephens *et al.* 1988), PMN infiltration (Schollmeier *et al.* 1990), increase in respiratory burst (Leff *et al.* 1993) and an increase in BALF cytokines such as interleukin-1 (IL-1) (Debs *et al.* 1988). Indeed EC permeability and PMN degranulation have been shown to be directly inducible by TNF- α (Horvath *et al.* 1988; Lo *et al.* 1992; Tracey *et al.* 1986) and it can mediate effects such as PMN influx and degranulation indirectly through the actions of another cytokine, IL-8 (Willems *et al.* 1989; Brandt *et al.* 1992). Characteristics of more chronic inflammatory lung diseases, such as pulmonary fibrosis, granuloma formation and T cell infiltration can also be mediated by TNF- α either directly or indirectly. Indeed, studies have demonstrated a protective effect of anti-TNF antibodies in animal models of pulmonary fibrosis, highlighting the important role of TNF- α in the pathogenesis of fibrotic lung injury (Piguet *et al.* 1989; Piguet *et al.* 1990). These known properties of

TNF- α underline the importance of this cytokine, and the paramount need to regulate its production and/or activity. There are many in-built regulatory mechanisms in the cytokine network, including production of inhibitory molecules. With reference to TNF- α , these include the cytokines IL-10 (Fiorentino *et al.* 1991), IL-6 (Aggarwal and Pocsik, 1992), IL-4 (Essner *et al.* 1989) and IL-13 (Yanagawa *et al.* 1995) which inhibit TNF- α production by many cells including AMs, and TNF soluble receptor (TNF-R). Two types of soluble receptor have been identified; p55 or type I and p75 or type II (Engelmann *et al.* 1990) and are identical to the membrane-bound forms of TNF receptor. p55 is thought to have a major role in cell signalling and is ubiquitous, whereas the p75 receptor may predominate on the surface of immune cells (Joyce *et al.* 1995; Kalthoff *et al.* 1993; Krönke *et al.* 1990). Both are capable of inhibiting biological activity (Leeuwenberg *et al.* 1994) although there is the possibility that p75 increases the stability of the TNF molecule, prolonging its presence and activity at the site of inflammation (Mohler *et al.* 1993; Aderka *et al.* 1992). Structural instability is another regulatory mechanism. The TNF molecule is most potent as a trimer, since this form permits cross-linking of the membrane bound p55 receptor resulting in enhanced signalling (Aderka *et al.* 1992). TNF- α only maintains its trimeric structure for a matter of minutes, dissociating to dimers and the inactive monomer (Corti *et al.* 1992). There is also in-built instability in the TNF- α mRNA. Many LPS-inducible cytokines, including TNF- α contain an octameric sequence of nucleotides at the 3' untranslated region (3' UTR). This sequence is preferentially hydrolysed by ribonucleases so restricting the storage of cytokine transcripts (Han *et al.* 1991a; Beutler *et al.* 1988).

Regulatory mechanisms for TNF- α appear to be insufficient in inflammatory lung diseases, and it has been suggested that anti-TNF antibody treatment may be efficacious. However, proof of such mechanisms is difficult to attain, not only because TNF- α is a very early mediator and thus the therapeutic window is very narrow, but also because it is not solely responsible for the injury. Other implicated cytokines include IL-1- β , which has a high degree of cross-over in terms of activity with TNF- α (see section 1.3.2), and IFN- γ . IFN- γ is thought to be very important in acute lung injury. Indeed anti-IFN antibodies can protect against lethality in a mouse model of lung injury in a similar fashion to anti-TNF antibodies (Doherty *et al.* 1992). Also in the Schwartzman reaction,

where LPS administration, followed at 24 hours by TNF- α administration led to the onset of fatal shock, neutralisation of IFN- γ with antibodies prevents lethality (Billiau, 1988). It must also be remembered that TNF- α is in itself a beneficial cytokine, important for host defence and microbial cell killing, as in *Listeria monocytogenes* (Nakane *et al.* 1989) and in the prevention of autoimmunity by its regulation of a subset of B cells implicated in autoantibody production (Ishida *et al.* 1994). Thus total removal of TNF- α during an inflammatory response may lead to some undesired consequences. Therefore a more thorough understanding of regulatory mechanisms in inflammation could lead to the design of more appropriate therapeutic manoeuvres.

1.3.2 Interleukin-1- β

The cytokine network is characterised by a certain amount of redundancy, or overlap between various mediators. This is certainly the case for IL-1- β and TNF- α . Not only are they able to induce production of each other on AMs and PBMs (Bachwich *et al.* 1986a; Nathan, 1987), but they also overlap in terms of fibroblast proliferation (Kohase *et al.* 1987), EC proliferation (Thornhill and Haskard, 1990), procoagulant activity (Bevilacqua *et al.* 1986) and production of other cytokines such as IL-8 (Matsushima and Oppenheim, 1989) and IL-6 (Jablons *et al.* 1989). However, they do differ in some circumstances, since IL-1- β is unable to elicit the PMN respiratory burst, or produce the same degree of shock in animal models. It remains the case, however that these two structurally distinct and unrelated cytokines share a number of functions. This redundancy at first glance seems perplexing, but the existence of overlap may enhance the efficiency of the cytokine response in critical situations and permits synergistic relationships which can cause enormous amplification of the cytokine cascade should circumstances warrant it.

1.3.3 The Chemokines

Movement of effector cells from the peripheral circulation to the tissues is a key event in the establishment of an inflammatory response. Recent studies have identified the existence of a superfamily of related cytokines, designated the chemokines, which can be

divided into two groups on the basis of conserved cysteine residues in the peptide sequence; C-C and C-X-C. IL-8 is one of the best characterised of the chemokines, and has been implicated in lymphocyte homing and PMN infiltration (Baggiolini *et al.* 1989). It is also capable of triggering the respiratory burst (Serra *et al.* 1994). TNF- α can initiate the release of IL-8 from EC and AMs (Brown *et al.* 1993; Cohen *et al.* 1988) and many of the indirect effects of TNF- α on PMNs are likely to be mediated by IL-8. PBM influx is thought to be under the control of two other chemokines, macrophage inflammatory protein (MIP-1- α) and monocyte chemoattractant peptide (MCP-1). Both are products of AMs (Schall *et al.* 1993; Brieland *et al.* 1995), as is the chemokine RANTES (Folkard *et al.* 1995) which is a T cell and eosinophil chemoattractant. TNF- α can trigger the release of MIP-1- α and MCP-1 from AMs, but its relationship to RANTES is unknown. It is apparent however, that chemokine production does lag behind TNF- α production, thus suggesting a possible role for TNF- α in triggering the cytokine network, which can then lead to the establishment of a full inflammatory response.

1.3.4 Interleukin-10

IL-10 was first described as a cytokine synthesis inhibitory factor (CSIF) and was initially discovered to be a product of T cells (human and cell lines) (Yssel *et al.* 1992; Ralph *et al.* 1992), maximally synthesised at 24-48 hours after T cell activation. In mouse models it was defined as a product specifically of Th2 cells, with an ability to inhibit Th1 cytokine synthesis (Yssel *et al.* 1992) and thus had similar properties to IL-4 (Oswald *et al.* 1992a; Sieling *et al.* 1993; Mulligan *et al.* 1993), but a recent study on human T cell populations suggests that Th1 and Th2 subsets cannot be distinguished on the basis of their production or response to IL-10 (Del Prete *et al.* 1993). The ability of IL-10 to inhibit pro-inflammatory cytokine (TNF- α , IL-1 and IL-6) release by human peripheral blood mononuclear cells (PBMCs) is well documented (Wang *et al.* 1994; De Waal Malefyt *et al.* 1991b; Fiorentino *et al.* 1991; Ralph *et al.* 1992). It has also been demonstrated to inhibit TNF- α and IL-8 release (Kasama *et al.* 1994; Cassatella *et al.* 1993) and MIP-1- α production (Kasama *et al.* 1994) by PMNs. IL-10 can also prevent T cell proliferation by inhibiting accessory cell function in APCs (Ding and Shevach,

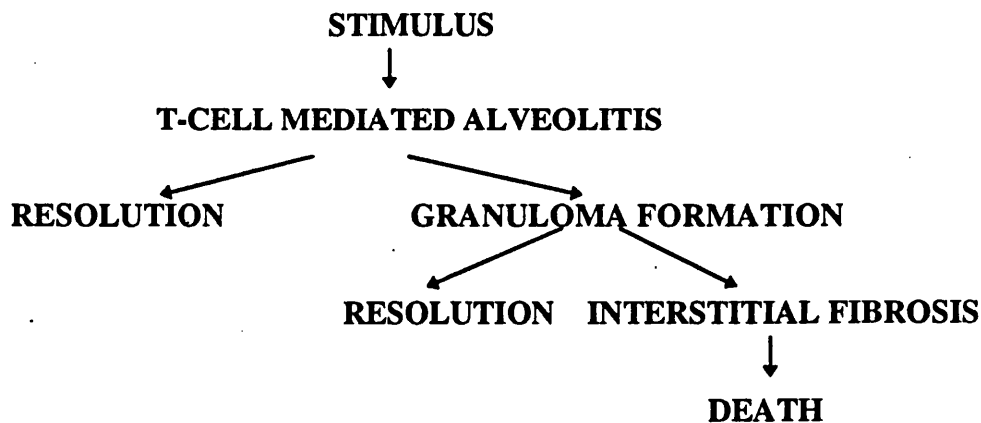
1992; de Waal Malefyt *et al.* 1991b), and it achieves this possibly by reducing class II MHC expression (Ding *et al.* 1993b) or down-regulating expression of B7, the CD28 ligand required for T cell activation (Enk *et al.* 1993). Interestingly, IL-10 appears to have no effect on dendritic cell accessory cell function (Macatonia *et al.* 1993). These anti-inflammatory properties of IL-10 generated much interest among those investigating inflammatory disease. IL-10 has been found to be elevated in septic shock (Marchant *et al.* 1994), ARDS (Donnelly *et al.* 1993), rheumatoid arthritis and osteoarthritis (Katsikis *et al.* 1994), and with reference to the latter, inhibition of IL-10 with antibodies elevated the pro-inflammatory cytokines in synovial fluid (Katsikis *et al.* 1994). Use of exogenous IL-10 has been demonstrated to prevent lethality in animal models of shock initiated by LPS infusion (Gerard *et al.* 1993; Howard *et al.* 1993; Smith *et al.* 1994) and the use of anti-IL-10 antibodies in one model using sub-lethal doses of LPS resulted in 30% lethality (Florquin *et al.* 1994). This effect may well be due to the inhibitory activity of IL-10 on TNF- α and IL-1. Interest in a putative regulatory role for IL-10 on TNF- α has been given further support by the finding that IL-10 can be produced by PBMs (Fiorentino *et al.* 1991; Wanidworanun and Strober, 1993; Abrams *et al.* 1992), possibly in response to LPS and TNF- α (Wanidworanun and Strober, 1993). Indeed, anti-TNF antibodies added to PBMC cultures reduced the production of IL-10, suggesting the possible existence of a sensitive autocrine regulatory loop in mononuclear cells. The fact that IL-10 is produced at least ten hours after TNF- α in mouse macrophages supports this hypothesis (Frei *et al.* 1993). Naturally, IL-10 does have relationships with other cytokines. It is thought that many of the anti-inflammatory effects of IL-10 could be due to inhibition of IFN- γ production by Th1 cells, so inhibiting the IFN macrophage activation pathway. IL-10 has also been frequently studied alongside IL-4 (see section 1.3.5) since they are both Th2 products. It should also be pointed out however, that IL-10 is not merely inhibitory in its activity. Protein synthesis up-regulation has been observed in response to IL-10, notably elastin (Reitamo *et al.* 1994) which could be important in healing, and the regulatory proteins IL-1 receptor antagonist (IL-1ra) and soluble TNF receptor (TNF-R) (Joyce *et al.* 1995).

1.3.5 Interleukin-4 and Interleukin-13

IL-4 has frequently been described as an anti-inflammatory cytokine, with the ability to inhibit the production of IL-1 and TNF- α by AMs (Nishioka *et al.* 1991). A product of Th2 cells, it can inhibit the production of IFN- γ by Th1 cells, a property in common with IL-10 (Paul, 1987). There have been a number of studies exploring the relationship between IL-10 and IL-4. A putative synergy has been suggested to exist in the establishment of type IV hypersensitivity (Powrie *et al.* 1993) which may be important in sarcoidosis (see section 1.4). However, another study on *Mycobacterium leprae* demonstrated an antagonistic relationship, with IL-4 inhibiting IL-10 production by Th2 cells (Sieling *et al.* 1993). Another cytokine closely associated with IL-4 is IL-13. This T cell product has been found to share many functions with IL-4 (Herbert *et al.* 1994) and this is due at least in part to shared receptor components (Aversa *et al.* 1993). IL-13 has been demonstrated to down-regulate TNF- α production by human AMs and PBMs (Yanagawa *et al.* 1995; McKenzie *et al.* 1993), but its relationship to IL-10 and pulmonary inflammation remains to be established.

1.4 Sarcoidosis

Sarcoidosis is a chronic systemic disorder of unknown aetiology, characterised by the accumulation of inflammatory cells, most notably T cells and mononuclear phagocytes, into the tissues, and the development of non-caseating epithelioid granuloma with concomitant tissue injury developing in a minority of cases (Walters and du Bois 1995). Currently the incidence varies between three and fifty cases per hundred thousand in developed countries (Walters and du Bois 1995). Any part of the body can be affected, including the skin, where erythema nodosum may be the presenting symptom, and the eyes, where uveitis is often present (Walters and du Bois 1995). The lung however, is a favoured site; ninety per cent of sarcoidosis patients present with a pulmonary manifestation and twenty per cent will progress to some degree of pulmonary fibrosis. Of these cases, fifty per cent will die as a result of respiratory failure. The remainder of cases will either demonstrate a halt progression or resolve spontaneously (Crystal *et al.* 1981b).



1.4.1 Aetiology

Sarcoidosis may be chronic or acute in nature, and the factors determining the likely progression of disease remain unknown (Walters and du Bois 1995). The actual cause of sarcoidosis remains contentious. It is likely that antigen recognition is important in the formation and maintenance of granulomas (Walters and du Bois 1995), but no specific causative organism has been identified. This is in contrast to other granulomatous diseases such as schistosomiasis (Thorne and Mazza, 1991) (*Schistosoma mansoni*) and tuberculosis (*Mycobacterium tuberculosis*) (Rook *et al.* 1987), where pathogenicity has been established. It remains to be established whether sarcoidosis is caused by a specific pathogen or is an exaggerated response to common-place non-pathogenic organisms/antigens. The blood of sarcoidosis patients has been found to contain elevated serum immunoglobulins and immune complexes as well as autoantibodies (Semenzato, 1986; Walters and du Bois 1995). This may suggest a failure of immune regulation, with a non-specific antigen acting as the trigger. The genetic element of the disease supports the likelihood of an inflammatory, rather than infectious disease. There is a higher dual incidence in monozygotic than dizygotic twins, and there is also a suggested MHC component which is hereditary, with HLA-B8, A1, CW7 and DR3 pre-disposing to acute sarcoidosis and HLA-B13 more prevalent in the chronic form of the disease (Semenzato, 1986). In addition there is circumstantial evidence of a racial element; African Americans have a higher risk of developing sarcoidosis than their Caucasian counterparts (Sharma and Johnson, 1988). Despite this evidence, the case is still strong for an infectious cause. Some studies have proposed the existence of mycoplasma in the

lung of sarcoidosis patients, although it is unclear whether it is as a pathogen or passenger (Thomas and Hunninghake, 1987). There is also the Kveim-Siltzbach test (Munro and Mitchell, 1987) where human sarcoid spleen extract injected intra-dermally into sarcoidosis patients results in localised granuloma formation within four to six weeks. Interestingly, there is no effect in normal individuals suggesting that the sarcoidosis patients do have an abnormal response to whatever agent is contained within the splenic extract, be it immunological or antigenic in nature.

1.4.2 Pathology

Sarcoidosis is characterised by the presence of multiple non-caseating granulomas. In the lung they are found mainly in the alveolar septa (Crystal *et al.* 1981b), but they can also be found in the walls of the bronchi, and the pulmonary arteries and veins. In the broadest terms, granulomas are collections of mononuclear inflammatory cells at an inflammatory locus. The cells are mostly epithelioid in nature, but there are also some multi-nucleated giant cells present (Crystal *et al.* 1981b). Epithelioid cells are believed to be derived from the monocyte/macrophage lineage and are strongly HLA-DR positive. They also contain large amounts of endoplasmic reticulum suggesting that they are active secretory cells (Semenzato, 1986). The Kveim-Siltzbach test has shed light on the development of granulomas and suggests that the mononuclear cells arrive at a particular site (where antigen is present, possibly) and mature into epithelioid cells. During maturation the T helper CD4 cells infiltrate, with some CD8 cells and together with AMs and newly arrived monocytes, form a perimeter around the epithelioid cells (Munro and Mitchell, 1987). The granuloma is surrounded by extracellular material, or reticulin, which consists of a collagenous matrix. This rim of collagen is implicated in the fibrotic end-stage disease, which may be ultimately fatal (Crystal *et al.* 1981b). Indeed the behaviour of the dynamic granuloma is pivotal to the pathogenesis of sarcoidosis since it is this structure which is responsible for the alveolar structure derangement and the consequent pulmonary complications, such as breathlessness and the fibrosis of end-stage sarcoidosis (Crystal *et al.* 1981b; Semenzato, 1986).

The BALF in sarcoidosis has been shown to correlate closely with the lung histology from the interstitium (Hudspeth *et al.* 1987). Attention has been drawn to the role of

AMs in this disease due to increased recovery of these cells in the BALF (Hunninghake and Crystal, 1981). This may be due in part to enhanced proliferation in the lung compartment, since elevated GM-CSF, which is mitogenic for AMs has been found to be elevated in sarcoidosis (Itoh *et al.* 1990; Itoh *et al.* 1993). In addition, there is evidence for DNA synthesis in AMs, indicative of cell division (Lin *et al.* 1989). However there is evidence that the majority of the additional AMs are newly arrived PBMs which have entered the lung in response to chemotactic signals such as MIP-1- α and MCP-1 (Crystal *et al.* 1981b). AMs in sarcoidosis have an immature morphology when compared to normal AMs (Hance *et al.* 1985). They also possess a number of monocyte antigens such as OKM1, which are generally lost upon maturation into macrophages (Hance *et al.* 1985). Enhanced clustering of sarcoidosis AMs with lymphocytes has been observed, which is a feature of monocyte antigen presentation. Indeed AMs, which are usually poor APCs are potent in the autologous mixed lymphocyte reaction when derived from patients with sarcoidosis (Lem *et al.* 1985; Gant *et al.* 1991). This is in part a reflection upon enhanced HLA-DR expression in these cells. This function is thought to be crucial in the establishment of granulomas. It is thought that the first step in granuloma formation is the phagocytosis of an antigen by AMs. In *In vitro* studies with macrophages, ingestion of antigens which are difficult to fully degrade, and are therefore persistent, leads to the development of granulomas (Dannenberg, 1975). These antigens are processed to some degree and presented to T helper cells in conjunction with class II MHC, and their persistent presentation leads to an ongoing AM/CD4 response which is thought to establish and maintain the granulomas through cytokine release. Indeed enhanced cytokine release is also a feature of sarcoidosis AMs, with elevations in IL-1- β (Steffen *et al.* 1993; Yamaguchi *et al.* 1988; Hunninghake, 1984), IFN- γ (Robinson *et al.* 1985), and TNF- α (Steffen *et al.* 1993; Terao *et al.* 1993; Foley *et al.* 1992) production by these cells. Sarcoidosis AMs also produce elevated levels of oxidant species, which may be important in the interstitial scarring, or fibrosis seen in a minority of patients (Baughman *et al.* 1988).

Clearly the T cell is very important in the pathogenesis of sarcoidosis, and they may account for up to sixty per cent of the effector cells in sarcoidosis BALF (Campbell *et al.* 1986), compared to less than eight per cent in normal BALF (Crystal *et al.* 1981b). Ninety per cent of the T cells are CD4, compared to sixty five to ninety per cent in the

normal lung (Crystal *et al.* 1981b). The balance of T cell subsets is altered in the disease state in favour of T helper cells (Hunninghake and Crystal, 1981). Indeed a CD4:CD8 ratio greater than five (Steffen *et al.* 1993) has been used to distinguish active sarcoidosis from the inactive disease by some investigators. The T cells in the lung contrasts sharply with those in the periphery, where T cell anergy has been observed (Hudspith *et al.* 1987; Hunninghake and Crystal, 1981), and the population is balance in favour of CD8 T cells. This accounts for the negative response to the tuberculin antigen skin test, used previously as a diagnostic indicator (Semenzato, 1986). Pulmonary T cells in sarcoidosis have a higher state of activation as determined by the presence of the activation marker CD69 (Crystal *et al.* 1981b) and their enhanced production of Th1 cytokines such as IFN- γ . This demonstrates that sarcoidosis is a compartmentalised disease. It is widely believed that cross-talk between AMs and T cells is fundamental to the inflammatory response in sarcoidosis, and that resolution of the disease is reliant upon T cell suppression by AMs (Ina *et al.* 1990). AMs are thought to have a major role in attracting effector T cells into the lung in sarcoidosis. Their importance has been highlighted by studies on sub-populations of AMs which have demonstrated a shift in phenotype away from the phagocytic tissue macrophage towards the accessory cell and T cell suppressor phenotypes (Campbell *et al.* 1986). These cells have been shown to promote and down-regulate T cell responses respectively (Spiteri and Poulter, 1991) and their balance may be crucial to the pathogenesis of the disease (Spiteri *et al.* 1992). Studies have been carried out on the functional properties of these distinct subsets, but as yet there has been no published data regarding their cytokine profiles, so it cannot be determined whether distorted AM populations are implicated in the changes in cytokine release exhibited by heterogeneous sarcoidosis AMs.

1.5 Cryptogenic fibrosing alveolitis

Cryptogenic fibrosing alveolitis (CFA), known as idiopathic pulmonary fibrosis (IPF) in the USA, is characterised by an influx of inflammatory cells into the alveolar space and progressive fibrosis of the pulmonary interstitium and air spaces (Crystal *et al.* 1984). Lone CFA presents with a pulmonary disorder, but fibrosing alveolitis is often present in patients with other diseases such as systemic sclerosis (Miller, 1990) and rheumatoid

arthritis (Sewell and Trentham, 1993) and the characteristics of the fibrosis are indistinguishable from CFA. CFA occurs spontaneously in three to five per hundred thousand population in developed countries, the onset is usually between forty and seventy years of age (Turner-Warwick *et al.* 1980). Once established, the disease is relentless in its progression, usually resulting in death from respiratory insufficiency within five years of the first symptoms (Stack *et al.* 1972).

1.5.1 Aetiology

The cause of CFA, like sarcoidosis, is unknown although numerous infectious causes have been alluded to. Persistent viral infection has been proposed as a trigger for the inflammatory process in CFA (Kawanami *et al.* 1979) since the onset of disease can sometimes be associated with flu-like symptoms. Also, a recent study has demonstrated the existence of Epstein Barr Virus in seventy per cent of type II pneumocytes (surfactant-secreting pulmonary cells) of CFA patients whereas it was only present in nine per cent of pneumocytes in control subjects (Egan *et al.* 1995). However it remains to be proven whether there is a definitive association between CFA and a pathogenic organism. Genetic factors may well be important, since there is a familial CFA which is clinically indistinguishable from the non-familial form of the disease, although the age of onset is much lower (Barzo, 1985). This genetic predisposition appears to be linked to an increased incidence of HLA-B8, B12, Dw6 and DR2 antigens and a decrease in the expression of HLA-Dw3 (Libby *et al.* 1983). This link is further strengthened by the association of fibrosing alveolitis with genetic disorders such as neurofibromatosis, tuberous sclerosis and Gaucher's disease (Walters and du Bois 1995). An important trigger for the inflammatory process in CFA appears to be immune complexes, which have been found to be elevated in the BALF and plasma of CFA patients (Libby *et al.* 1983). These are ingested by scavenging AMs, triggering cytokine release, a vital step in the initiation of the inflammatory response, and they can also trigger PMN degranulation directly (Zhang *et al.* 1992). It is not clear however, whether these complexes arise as a result of a specific pathogen, or they are a manifestation of an altered immune state responding to a harmless environmental agent. Not all environmental agents are harmless however, and fibrosis can arise from this type of injury. Pulmonary fibrosis is a feature of

asbestosis and silicosis and is clinically very similar to CFA (Crystal *et al.* 1984). Fibrosis can also arise iatrogenically, the cardiac drug amioderone can elicit this response (du Bois, 1993), as can the anti-neoplastic agent bleomycin, which is administered intra-tracheally to produce an animal model of pulmonary fibrosis (Tomioka *et al.* 1989; Thrall *et al.* 1982).

1.5.2 Pathology

Pulmonary fibrosis is debilitating due to the derangement and progressive loss of alveolar structures as a result of excess collagen deposition. Collagens account for sixty per cent of the pulmonary extra-cellular matrix, and are interspersed with elastin, structural glycoproteins such as fibronectin, and thrombospondins important in wound healing (Campa *et al.* 1993).

The mechanisms which lead to the development of pulmonary fibrosis in CFA are partially understood. One of the most important events is the establishment of a neutrophil-mediated alveolitis. It is thought likely that intra-pulmonary cellular activation leads to production of chemokines such as IL-8, which attracts PMNs into the alveolar spaces from the periphery (Baggiolini *et al.* 1989). Once arrived in the lung the PMNs degranulate, releasing a host of proteolytic enzymes including collagenases (Libby *et al.* 1983) which can inflict tissue damage directly. There is also the respiratory burst, a property of activated AMs and PMNs, whose products are extremely toxic to the local environment. Such toxicity leads to further tissue damage and the inevitable scarring. In normal circumstances, however the inflammatory response is finite, and following removal of the pathogen or irritant resolution of inflammation ensues. This does not appear to be the case in CFA. The alveolitis is maintained by the interplay of cytokine mediators and inflammatory cells. Ongoing inflammatory events lead to progressive fibrosis and eventually death. The role of the AM appears to be crucial in CFA, since it is able to release cytokines such as TNF- α and IL-10 which have the ability to promote and dampen down the inflammatory response respectively. Indeed the AM in CFA BALF is abnormal, with increased capacity to produce TNF- α , IL-8 and MIP-1- α (Standiford *et al.* 1993) and enhanced superoxide production (Schaberg *et al.* 1993). TNF- α itself may be an important mediator in CFA since apart from its ability to trigger PMN

degranulation and the respiratory burst in AMs (Klebanoff *et al.* 1986) it is also mitogenic for fibroblasts (Kohase *et al.* 1987) and can increase their deposition of type I and III collagen (Campa 1993). TNF- α can also stimulate release of IL-8 from AMs, which is vital for the PMN infiltration, and correlates with the severity of CFA (Carre *et al.* 1991).

1.6 Adult respiratory distress syndrome (ARDS)

ARDS was first described in 1967 by Ashbaugh as a disease presenting itself in critically ill patients on the intensive therapy unit. It has since been established that ARDS develops in 2.5% of all ITU admissions in the United Kingdom, or 0.045 cases per thousand population (Webster *et al.* 1988). It has been recognised that there are pre-disposing risk factors for the disease, notably severe trauma and sepsis and that ARDS develops in fourteen per cent of patients with these risk factors (Webster *et al.* 1988). The syndrome is characterised by respiratory insufficiency, mediated by an acute inflammatory process and culminating in severe pulmonary oedema and fibrosis. Death ensues within days or weeks in more than fifty per cent of cases (Hert and Albert, 1994). These processes can occur however, in other parts of the body, such as the kidney or liver, and in this respect ARDS can be considered the pulmonary manifestation of multiple organ failure (Fothergill *et al.* 1987; Hyers *et al.* 1987).

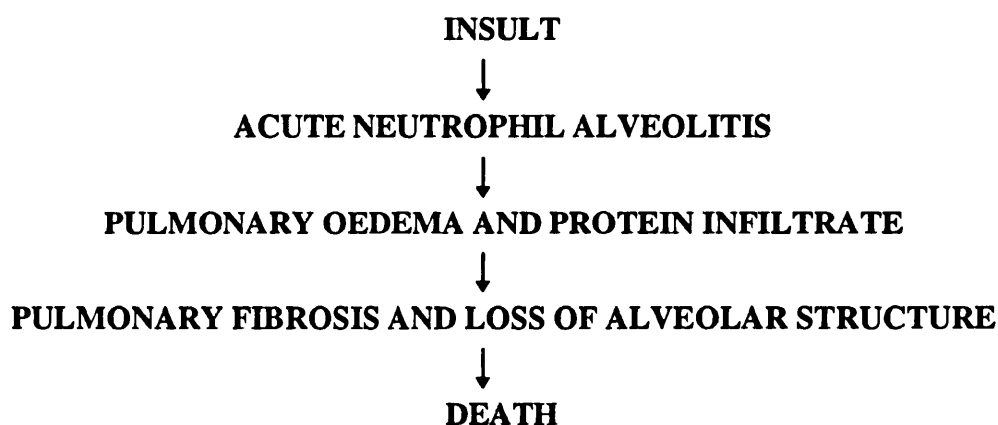
1.6.1 Aetiology

It has been recognised that certain conditions pre-dispose to the development of ARDS. Sepsis syndrome appears to invoke the highest risk of ARDS development, with more than forty per cent of sepsis patients succumbing to ARDS (Fothergill *et al.* 1987). Gram negative sepsis patients are particularly prone to ARDS progression, and it is thought that endotoxin/LPS may be a key trigger for the catastrophic inflammatory process seen in ARDS. The disease can also arise from perforated bowel and acute pancreatitis, where the likely existence of a systemic infection is very high. Similarly multiple trauma, resulting from road traffic accidents, gun shot injuries and the breaking of two or more long bones also pre-disposes, once again possibly as the result of serious infection, or by

massive activation of the coagulation cascade (Pepe *et al.* 1982). However ARDS can also arise in circumstances where an infectious cause is unlikely. These include transfusion of more than eight units of blood, where activation of peripheral blood inflammatory cells may be a feature. Direct lung injury can also lead to ARDS, examples of which are aspiration of gastric contents and near-drowning episodes. Pulmonary infections such as pneumonia are also pre-disposing risk factors.

1.6.2 Pathology

Death from ARDS usually occurs as a direct result of respiratory failure, arising from a sequence of events:

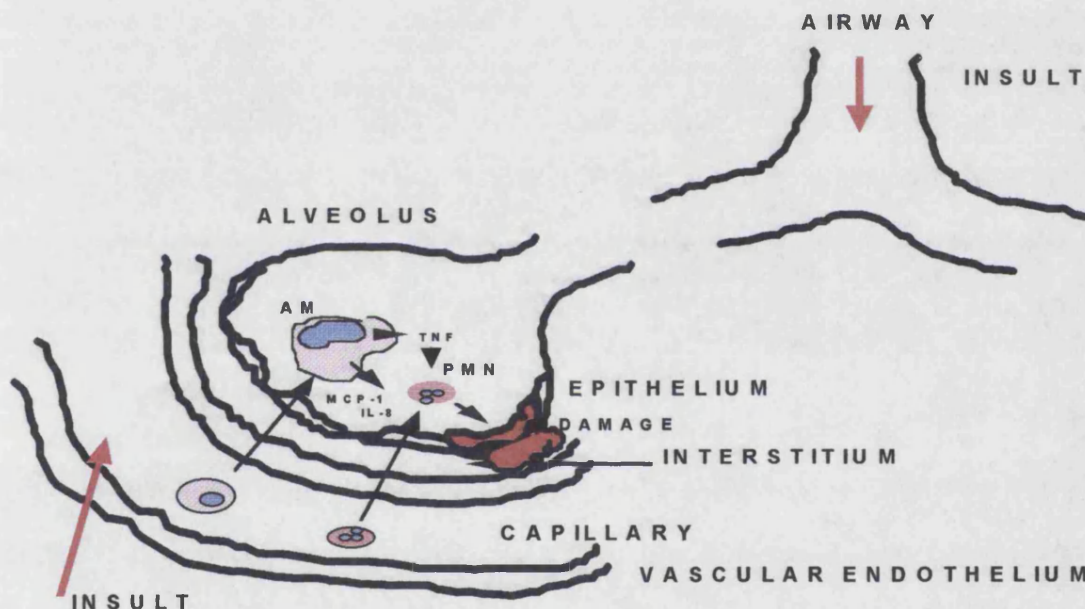


In contrast to CFA, this process is not necessarily relentless. It may stop at any time and can lead to complete resolution (Elliott *et al.* 1987).

Since ARDS is frequently associated with gram negative sepsis, one of the primary triggering events is considered to be endotoxin, a protein shed or released from lysed bacterial cell walls. The potential of endotoxin to invoke an inflammatory response with the hallmarks of ARDS has been demonstrated in animal models of acute lung injury utilising endotoxin (also known as lipopolysaccharide (LPS)) infusion (Rose *et al.* 1994). LPS is capable of triggering the release of monokines such as IL-1 and TNF- α from macrophages and monocytes, and these cytokines have been found to be produced in high quantities by AMs from ARDS BALF (Suter *et al.* 1992). These mediators are thought to trigger an inflammatory cascade involving other cytokines such as IL-8, MCP-1, IL-10 and IFN- γ which can promote or regulate the inflammatory response.

These mediators are not only released in response to LPS, however. Direct trauma to the lung can trigger cytokine release from damaged epithelial cells lining the lung walls and these cytokines, including TNF- α and IL-1 can lead to AM activation and triggering of the inflammatory cascade. The inflammatory process in ARDS is markedly similar to CFA, and it is likely that mediators such as TNF- α and IL-8 are similarly important. Indeed a recent study by Donnelly and co-workers (1993) has indicated that high IL-8 is a prognostic indicator for ARDS in at risk subjects. Since the AM is a major source of both IL-8 and TNF- α it is likely that this cell is crucial to ARDS pathogenesis, and studies have demonstrated enhanced IL-8 activity in ARDS AMs (Donnelly *et al.* 1993), and an increased capacity to produce TNF- α (Tran Van Nhieu *et al.* 1993).

Figure 1.4 Proposed mechanisms of lung injury in ARDS



1.7 Aims of the Study

The inflammatory response is a fundamental defence mechanism, which if inappropriately controlled can lead to host tissue injury. TNF- α is a key inflammatory cytokine. Produced early in the inflammatory response, it has the ability to elicit production of other mediators within the cytokine network, with beneficial, but also potentially

deleterious effects. TNF- α itself has known pathogenic effects in the lung, including triggering of oxygen radical and proteolytic enzyme release by phagocytes, giving rise directly and indirectly to pulmonary oedema and the development of tissue fibrosis. It is also elevated in a number of inflammatory disease states. The major source of pulmonary TNF- α is thought to be the AM, a sentinel cell which represents the first line of defence in the lung. AMs are also known to be altered in pulmonary inflammatory disease. This thesis is based upon the hypothesis that AM-derived cytokine regulation is abnormal in pulmonary inflammatory disease and has focused specifically on the regulation of AM-derived TNF- α . The aims were as follows:

- (1). To determine the profile of TNF- α release in AMs and PBMs from normal subjects and to examine the regulatory mechanisms involved in its production and bioactivity, specifically in relation to IL-10 and TNF-R, and to establish whether these mechanisms were lung-specific or extended to the periphery.
- (2). To examine the relationship between AM and PBM-derived TNF- α , TNF-R and IL-10 in subjects in the three distinct inflammatory lung diseases sarcoidosis, CFA and ARDS, and to determine whether any alterations in their relationship were systemic or compartmentalised in nature.
- (3). To purify AM subpopulations from normal subjects and those with inflammatory lung disease in order determine any disease-specific phenotypic variation, or alteration in cytokine profile and what implications this may have for pulmonary TNF- α regulation.
- (4). To generate the AM phenotypes *in vitro* in PBMs and monocytic cell lines to permit more detailed study of the functional relationships and cytokine profiles of the subpopulations.

2. MATERIALS AND METHODS

2.1 Bronchoalveolar lavage.

Bronchoalveolar lavage fluid (BALF) was obtained from patients and normal volunteers consenting to undergo fiberoptic bronchoscopy. Prior to the procedure subjects were injected intramuscularly with 0.6 mg of atropine sulphate to reduce airway secretions, followed by intravenous sedation with 0-2 mg Alfentanil (Janssen) and 0-10 mg Midazolam (Roche). Topical lignocaine was administered to anaesthetise the upper airway. 0.9% sterile saline (Baxter Healthcare) was buffered to pH 7.4 with 8.4% sterile sodium bicarbonate and instilled into the right middle lobe or lingula in four 60 ml aliquots. The resultant BALF was then aspirated into glass bottles (siliconised to minimise macrophage adherence), kept at 4°C. Lavages collected for RNA analysis were immediately buffered at this point with 100 mls of chilled RPMI 1640 culture medium (Gibco) to maximise viability and thus prevent release of RNAses from lysed cells.

2.2 Alveolar macrophage preparation

Chilled BALF was strained through a single layer of sterile coarse gauze (Robinson's of Chesterfield) to remove mucus clumps, and the filtrate was then washed twice at 500 g, 4°C in complete media consisting of RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 0.5 µg/ml fungizone (all Gibco). If large numbers of erythrocytes were present in the resultant cell pellet they were lysed by treatment with ammonium chloride solution (see appendix). The pellet was then resuspended in complete media and viability assessed by 0.1% trypan blue exclusion. Cytospins of total BAL cells were taken at this point (see 2.6.1). The cells were then incubated at a concentration of 2×10^6 per ml in tissue culture petri dishes (Falcon) (no more than 3 mls per dish) for 2 hours at 37°C. The non-adherent cells were then discarded and the residual non-adherent cells removed by gentle rinsing in PBS. The adherent macrophages were removed from the dishes by gentle scraping with a cell scraper (Costar) and a cytospin taken for differential staining with Diff-Quick (Baxter-Dade) (see appendix).

Alveolar macrophages established >95% pure by this method were cultured in complete media at a concentration of 1×10^6 cells per ml, in microtitre plates (Nunc), 100 μ l per well. Cells were cultured for 24 hours unless stated and the viability rechecked with trypan blue. Supernatants were harvested after incubation in humidified 37°C incubators with 5% CO₂, and stored at -70°C until cytokine determination.

2.2.1 Preparation of AMs from ITU patients

Adherence could not be used to purify AMs from ITU patients since the neutrophils in the BALF were often in an activated state and thus adhered strongly to plastic. To surmount this problem the cells were purified by negative selection with magnetic beads (Dyna). Cells were incubated for 30 minutes at 4°C with mouse anti-human CD66b (The Binding Site) to remove granulocytes, and mouse anti-human CD3 (Dako) to remove T cells. The cells were then washed for 5 minutes in complete medium before incubation at 4°C with the magnetic beads (100 μ l of beads per 10^7 cells). The contaminating cells were then removed by exposure to a magnet for 1 minute. The remaining cells were then washed once in complete media for 5 minutes at 500 g and a cytopsin taken to assess purity. The resultant AMs were >95% pure, with >90% viability. They were then cultured as above.

2.2.3 Preparation of AMs for sub-population analysis

AMs were purified into 3 phenotypically distinct populations; RFD1-D7+, RFD1+D7- and RFD1+D7+ according to the protocol of Spiteri *et al.* (1992). AMs were adherence purified as described in section 2.2. The non-adherent cells were incubated with anti-CD3 magnetic beads for 30 minutes at 4°C, and the CD3 positive contaminating lymphocytes were removed by exposure to a magnet for 1 minute. The remaining cells were washed once in culture medium at 500 g, resuspended at 1×10^6 cells per ml and cultured for 24 hours. Cytopsins were taken to determine purity, and these cells were found to be >85% RFD1+D7- accessory AMs.

The adherent AMs, containing RFD1-D7+ and RFD1+D7+ cells were divided in to separate cell suspensions of 1×10^6 cell per ml. One population was exposed to

magnetic beads conjugated with anti-RFD1 antibody for 30 minutes at 4°C, with a bead to cell ratio of 10:1. The RFD1 positive cells were then removed by exposure to a magnet as described above, and the remaining cells washed, resuspended and cultured in culture medium as previously described. Cytospins were taken and immunocytochemical analysis revealed > 85% pure RFD1-D7+ tissue macrophages.

The remaining adherent AMs were layered on to a metrizamide gradient (14.5 g metrizamide dissolved in RPMI 1640, 5 mls used for each separation). This was centrifuged at 650 g for 10 minutes at room temperature. This process separated the less dense RFD1+D7+ suppressor AMs from the tissue macrophages, which pelleted at the bottom. The AMs at the interface were removed with a pastette and were washed, resuspended and cultured as above. Immunocytochemical analysis of this population revealed > 80% pure RFD1+D7+ suppressor AMs.

2.3 Peripheral blood monocyte preparation

Venepuncture was performed on healthy volunteers, and on bronchoscopy subjects prior to the procedure. Blood was collected into heparinised tubes, or for larger volumes into blood packs (Fenwall, Baxter Healthcare) pre-treated with 10 U of Heparin per ml of blood. Plasma was removed by centrifugation at 1000 g, 4°C for 6 minutes, and stored at -70°C until required. The remaining blood was diluted to twice the original volume with complete media, and carefully layered on to a Ficoll-Hypaque density gradient (specific gravity 1.077, Pharmacia). After 30 minutes centrifugation at 400 g, without braking, the resultant interface of mononuclear cells was removed, washed and resuspended in complete media supplemented with 10% heat-inactivated foetal calf serum (FCS) (Gibco). The cells were then allowed to adhere for 1 hour in tissue culture flasks (15 x 10⁶ cells per 75 cm² flask (Nunc)) at 37°C, 5% CO₂. The non-adherent cells were removed by rinsing with PBS. The adherent peripheral blood monocytes (PBM) were then removed by gentle scraping, washed and resuspended in complete media. Cells were >95% viable as assessed by trypan blue exclusion, and were >95% PBM as determined by morphology under diff-quick staining.

2.3.1 Twenty four hour monocyte culture

Isolated PBMs were resuspended at 1×10^6 cells per ml in complete media and cultured in 96 well microtitre plates, 100 μ l per well. They were co-incubated with the cytokine mediators of interest and the supernatants harvested after 24 hours. These were stored at -70°C until cytokine determination. Viability was checked with trypan blue after cell culture.

2.3.2 Monocyte-derived macrophage culture

Isolated PBMs were plated out on to tissue culture petri dishes (Falcon) in complete media supplemented with 10% FCS. They were incubated either alone or in the presence of a stimulus for up to 2 weeks. Media was changed every 3-4 days. Monocyte-derived macrophage (MDM) morphology was determined by taking cytospin samples (see section 2.6.1) at intervals over the 2 week period and immunostaining for macrophage markers (see sections 2.6.4/5).

2.4 Culture of cell lines

2.4.1 U937 cells

The human histiocytic lymphoma cell line U937 (Larrick *et al.* 1980) was cultured in complete media supplemented with 10% FCS at 37°C , 5% CO_2 up to a density of 1×10^6 cells per ml. The cells were then either diluted 1:5 with fresh media to maintain the culture or isolated for experimentation. Before isolation the cells were directed to a more macrophage morphology by overnight treatment with 10 nM phorbol myristate acetate (Sigma) (other maturation factors were also utilised (see Chapter 7)). The cells were then rinsed and detached by gentle scraping with a cell scraper before they were washed and resuspended in complete media at 1×10^6 cells per ml. They were then added to 96 well tissue culture microtitre plates, or 75 cm^2 tissue culture flasks and cultured for up to 24 hours with the appropriate mediators at 37°C , 5% CO_2 .

2.4.2 THP-1 cells

The human monocytic cell line THP-1 (Tsuchiya *et al.* 1980) was maintained in complete media supplemented with 10% FCS at 37°C, 5% CO₂ in a humidified incubator. They were fed at intervals of 4-5 days to maintain the optimum density of 1×10^6 cells per ml. Various maturation factors were utilised to achieve macrophage morphology for experimentation (see Chapter 7).

2.5 Measurement of protein by enzyme-linked immunosorbent assay (ELISA)

2.5.1 TNF- α

TNF- α protein was determined with a sandwich ELISA, whose antibodies were kindly donated by Dr Sue Stephens of Celltech, UK. These antibodies showed no cross-reactivity with other known cytokines, and their activity was not affected in the presence of human plasma or serum. ELISA plates (Nunc Maxisorp) were pre-coated with CB0006 mouse anti-human TNF monoclonal antibody at a concentration of 8 μ g/ml, in coating buffer (see appendix for constitution of ELISA buffers) and stored for up to a month before use. On the day of use TNF- α standards (NIBSC) (range 16 - 1000 pg/ml) and samples were diluted 1:4 in sample buffer and added, 50 μ l per well in duplicate. An inter-assay control TNF- α standard was added to each plate to assess variance. Inter-assay variance was less than 3 %. The plates were then incubated at room temperature for 1 hour with agitation. The plates were washed 3 times with wash buffer by a platewasher (Titertek) and patted dry. Rabbit anti-human TNF (WBRA-PG2) polyclonal antibody was then added 100 μ l per well at a concentration of 2 μ g/ml and incubated for a further hour with agitation. Following 3 washes, 100 μ l per well goat anti-rabbit IgG peroxidase (Sigma) diluted 1:500 was added and incubated for 30 minutes with agitation. After 4 washes with PBS 200 μ l of tetramethylbenzidine (TMB) substrate (Fluka) was added and left for 30 minutes in the dark to develop. The reaction was stopped by addition of 50 μ l of 1M H₂SO₄ and the plates read at 450 nm (reference wavelength 630 nm) on a platereader. Sample values were extrapolated from the standard curve, with a detection limit of 30 pg/ml TNF-alpha.

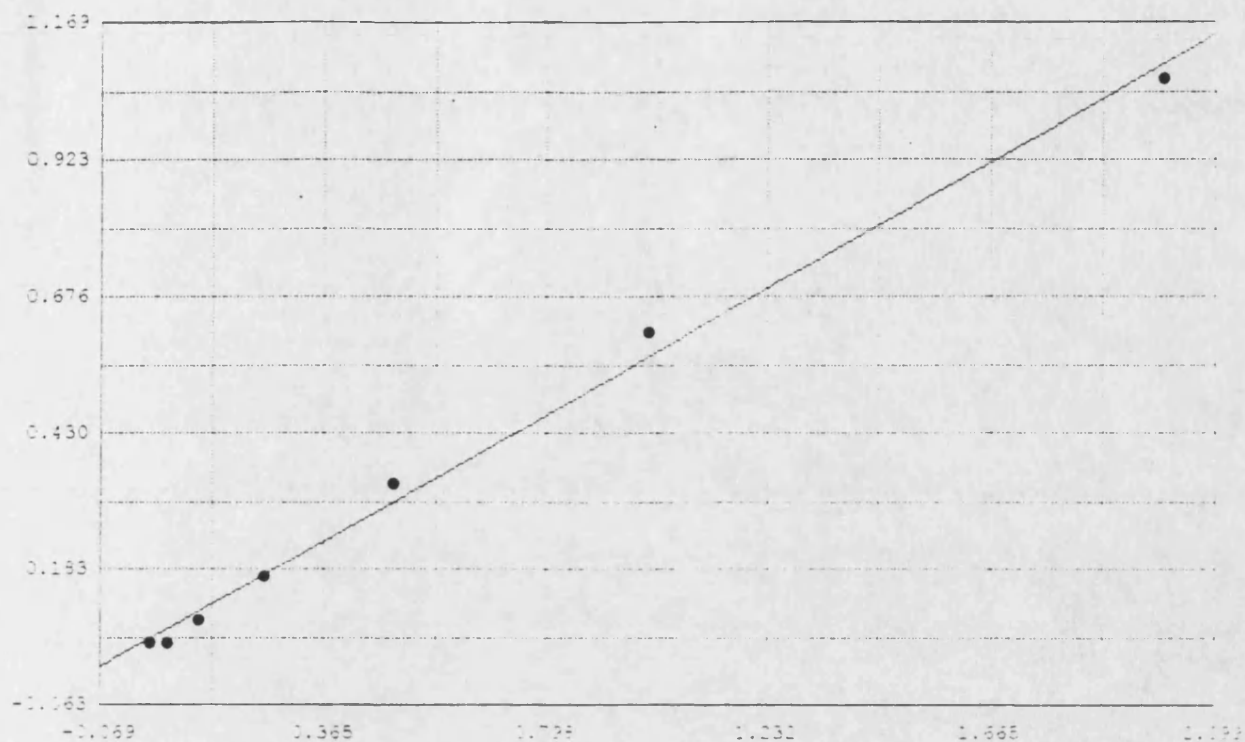
2.5.2 Interleukin-10

ELISA plates were coated with 1 µg/ml rat anti-human IL-10 monoclonal antibody (Pharmingen), 50 µl per well in coating buffer (see appendix), and left overnight at 4°C. The following day the plates were washed twice with wash buffer using a platewasher and patted dry. The wells were then blocked with 200 µl of PBS/10% FCS (sample buffer) and incubated at room temperature for 2 hours. The plates were subsequently washed twice, and IL-10 standards, range 1000 - 2 pg/ml (courtesy of DNAX) and samples diluted 1:4 in sample buffer were added, 100 µl per well. After a 4 hour incubation at room temperature the plates were washed 4 times with wash buffer. 2 µg/ml biotinylated rat anti-human monoclonal antibody (Pharmingen), diluted in sample buffer was then added, and incubated at room temperature for 45 minutes. Neither antibody showed cross-reactivity with other known cytokines, and their activity was not affected in the presence of human plasma or serum. After 6 washes with wash buffer a 1:400 dilution of avidin peroxidase (Sigma) was added, 100 µl per well and incubated for 30 minutes at room temperature. The plates were then washed 8 times and 100 µl of TMB substrate (see appendix) added and allowed to develop at room temperature in the dark for 10 minutes. The reaction was then stopped by addition of 50 µl of 1M H₂SO₄ and the plates read at 450 nm (reference wavelength 630 nm). Sample values were extrapolated from the standard curve, which gave a detection limit of 4 pg/ml.

2.5.3 p-55 TNF receptor (TNF-R)

ELISA plates were coated with 2 µg/ml IV4E monoclonal antibody (courtesy of Dr Terje Espevik, Trondheim, Norway) (Liabakk *et al.* 1993) in coating buffer (see appendix), 100 µl per well, and incubated for 12 hours at 4°C. This antibody showed cross-reactivity with other known cytokines, and its activity was not affected in the presence of human plasma or serum. The plates were then blocked with PBS containing 0.5% BSA for 1 hour at 37°C. The wells were then washed 3 times on a platewasher with PBS containing 0.1% Tween 20 (Sigma). Samples diluted 1:4 in sample buffer were then added, together with the recombinant p-55, 100 µl per well and the plates incubated overnight at 4°C. The following day plates were washed 4 times in wash buffer, and 100 µl of biotinylated TNF-α, 50 ng/ml was added to each well. The plates were incubated

Figure 2.1 TNF- α ELISA standard curve

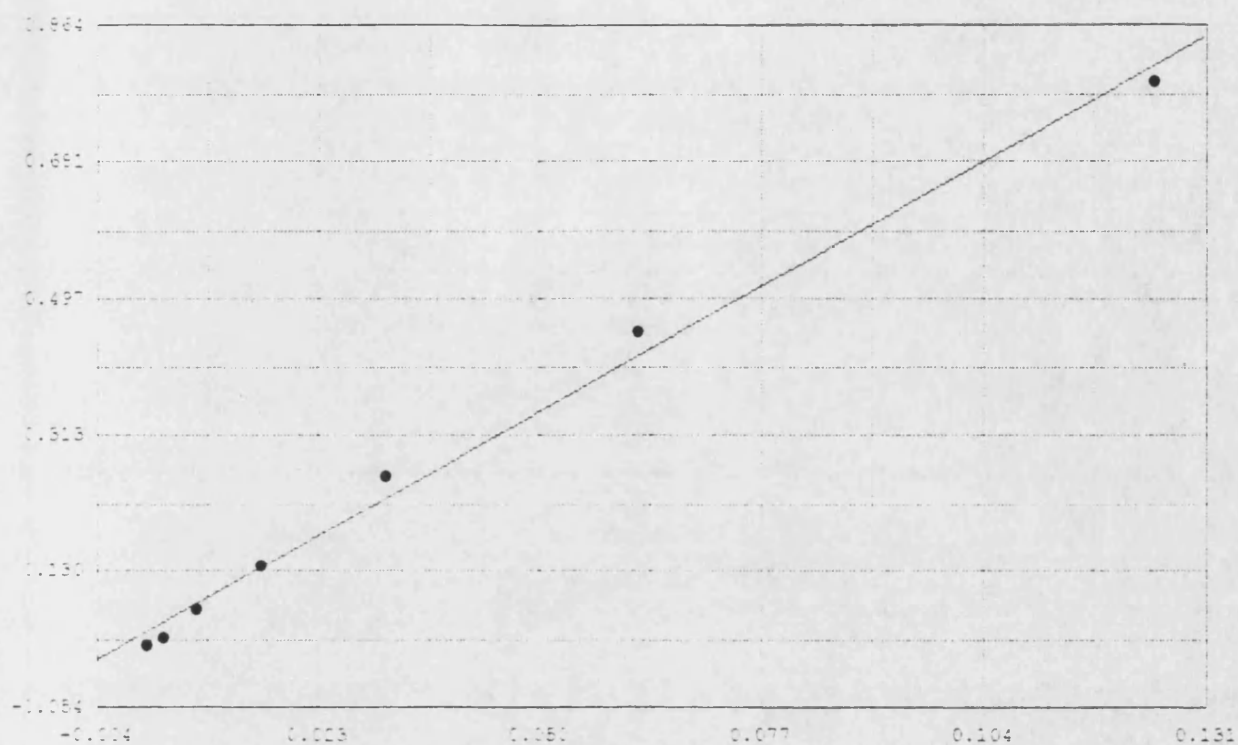


Unweighted Line Fit: OD vs. Conc. w/ table. $y=Ax+B$ $R^2=0.994$
 $A: 0.627$ $B: 0.043$

Standards:	S1	0.031 ng/ml
	S2	0.062
	S3	0.125
	S4	0.25
	S5	0.5
	S6	1
	S7	2

Detection limit = 0.03 ng/ml

Figure 2.2 IL-10 ELISA standard curve



Straight Line Fit: OD vs. Conc. w/ tails. $y=Ax+B$ RSquared = 0.993
 A: 0.010 B: 0.036

Standards:	S1	0.002 ng/ml
	S2	0.004
	S3	0.008
	S4	0.016
	S5	0.031
	S6	0.062
	S7	0.125

Detection limit = 0.004 ng/ml

for 1 hour at 37°C and then washed 4 times with wash buffer. The bound TNF was detected with streptavidin peroxidase 1:400 dilution, incubated for 30 minutes at room temperature, and the plates washed 4 times. TMB substrate was then added 200 µl per well and the plates left to develop in the dark for 15 minutes. The reaction was stopped with 50 µl of 1M H₂SO₄ and the plates read at 450 nm (reference filter 630 nm) on a platereader. Sample values were calculated from the resultant standard curve. The detection limit was 16 pg/ml

2.6 Detection of protein by immunohistochemistry

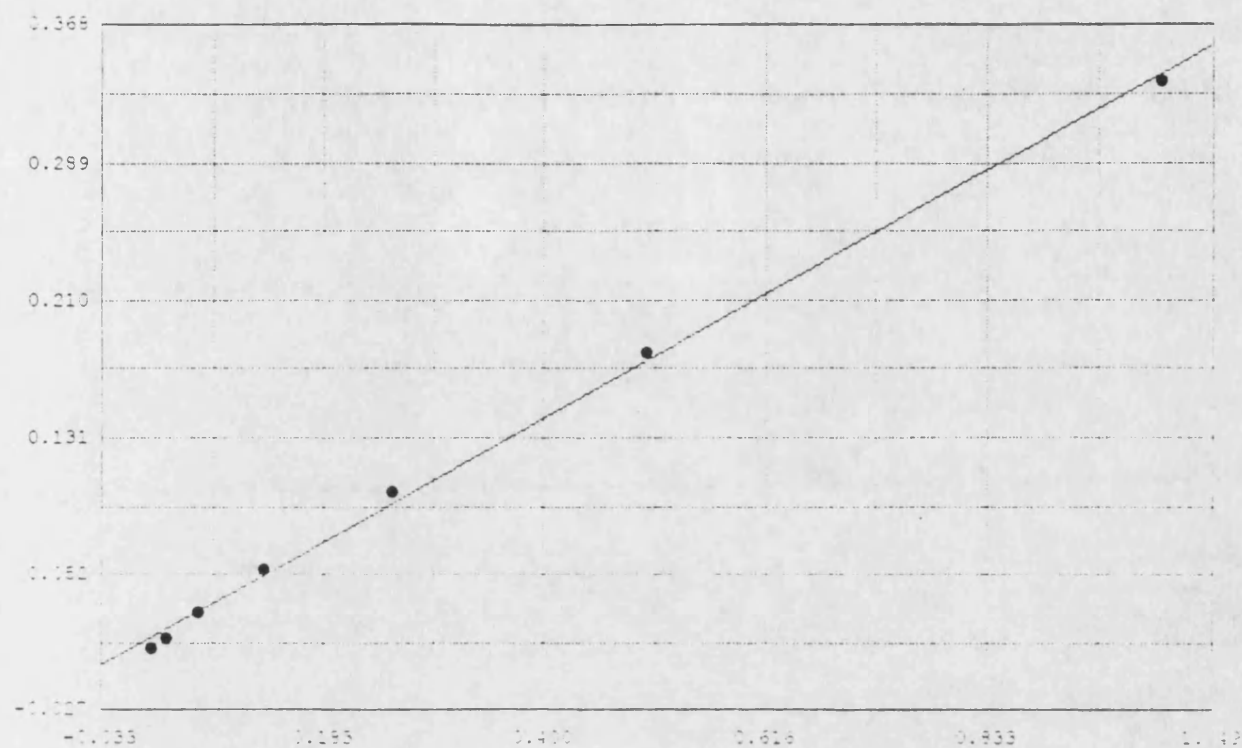
2.6.1 Cytospins

Cytospins were generated in order to assess cell suspensions for their purity e.g. AM and PBM purification, and also for intra-cellular staining of cytokines. In addition they were also utilised for phenotypic identification. The cells in question were resuspended in complete media at a density of 3.5×10^5 cells per ml and 200 µl was spun down on to each frosted glass slide (Berliner) (pre-treated with poly-L-lysine (Sigma)) in a cytocentrifuge (Shandon). After a 5 minute spin at 500 rpm the slides were removed and air dried for 30 minutes. If the cytospins were being used for purity assessment they were stained following air drying with Diff-Quick stain (see appendix). If the slides were destined for immunostaining they were wrapped in tin foil and stored at -70°C until required. Prior to the immunostaining procedure, the slides were defrosted and fixed in 1:1 Chloroform:Acetone for 10 minutes to permeabilise the membranes. The cell area was then enclosed within a hydrophobic film drawn with a PAP pen (Agar Scientific) to prevent leakage of antibody solution from the staining site. All immunostaining was performed in humidified chambers to prevent drying out of the cells.

2.6.2 TNF-α immunostaining

Fixed cytospins were blocked for 2 hours with 2% FCS at 37°C, before addition of the polyclonal rabbit anti-human TNF WBRA-PG2 antibody (courtesy of Celltech) at a 1:500 dilution. Rabbit IgG (Sigma) was added to control slides at the same concentration. Sections of human tonsil were used as a positive control. Following a 1.5

Figure 2.3 TNF-R ELISA standard curve



Straight Line Fit: OD vs. Conc. w/ tails. $y = Ax + B$ $R^2_{\text{adjusted}} = 0.998$
 A: 0.887 B: 0.010

Standards:	S1	0.016 ng/ml
	S2	0.031
	S3	0.062
	S4	0.125
	S5	0.25
	S6	0.5
	S7	1

Detection limit = 0.016 ng/ml

hour incubation at 37°C the slides were washed in PBS for 5 minutes. They were then incubated for a further 45 minutes at 37°C with goat anti-rabbit IgG peroxidase (Sigma), 1:500 dilution, before washing and addition of the 3,3'-diaminobenzidine (DAB) substrate (see appendix). This was incubated for 10 minutes at 37°C to develop a brown colour. The reaction was stopped by immersion in distilled water and the slides were counterstained with Mayer's haematoxylin (Sigma) for 5 minutes. They were then mounted in 9:1 glycerol:PBS and examined on a microscope at x 400 magnification. Five fields of view were observed and stain intensity was determined as follows:

0 = no stain, + = weak stain, ++ = moderate stain, +++ = strong stain

2.6.3 IL-10 immunostaining

Fixed test slides and the positive control slides (IL-10 transfected COS-7 cells) were blocked for 2 hours with PBS/2% FCS, before incubation for 1.5 hours at 37°C with biotinylated rat anti-human IL-10 monoclonal antibody (Pharmingen). Negative control slides were incubated with an identical concentration of rat IgG (Sigma). The slides were then washed as above before a 45 minute incubation with a 1:400 dilution of streptavidin peroxidase. Following a further wash the DAB substrate was added and left for 15 minutes in the dark to develop. Slides were analysed for positivity as above.

2.6.4 AM phenotypic analysis

AM phenotypes were determined by their expression of the markers RFD1 and RFD7. RFD1 is a cell membrane antigen identified by the RFD1 IgM mouse antibody, whereas the RFD7 mouse IgG1 antibody picks up an intracellular antigen. Both antibodies were supplied by Dr Len Poulter of the Royal Free Hospital. Fixed cytopins were incubated with a 1:5 dilution of each antibody in PBS (pH 7.6), with the control cells incubated with mouse immunoglobulins (Sigma) in PBS. Sections of human tonsil were used as a positive control. Following a 1 hour incubation the slides were washed for 2 minutes in PBS before incubation with a 1:30 dilution of fluorescein isothiocyanate- (FITC)-conjugated rabbit anti-mouse IgM (Sigma) and a 1:50 dilution of TRITC-conjugated goat anti-mouse IgG1 (Sera-Lab). These secondary antibodies were incubated for 45

minutes before being washed for 2 minutes in PBS. 9:1 glycerol:PBS was then dropped on to the slides which were then covered with a coverslip (Chance-Propper) and kept at 4°C in the dark before viewing under oil at x 1000 magnification on a fluorescent microscope (Nikon Optiphot). Fluorescence was compared to the control cells and monitored in 5 fields of view (see Plate 2.1).

2.6.5 CD68 immunostaining

CD68 is a pan-macrophage marker and was thus used to demonstrate the percentage of AMs on the cytopins. Briefly, cytopins were treated as above, but incubated with a mouse anti-human antibody to CD68 (Dako), at a 1:50 dilution. After a 1 hour incubation the slides were washed as above and then incubated with a 1:20 dilution of FITC-conjugated anti-mouse Ig's (Dako). Following a further 45 minute incubation the slides were washed, prepared and analysed as above (see Plate 2.1).

2.7 Northern Analysis

2.7.1 Cell culture

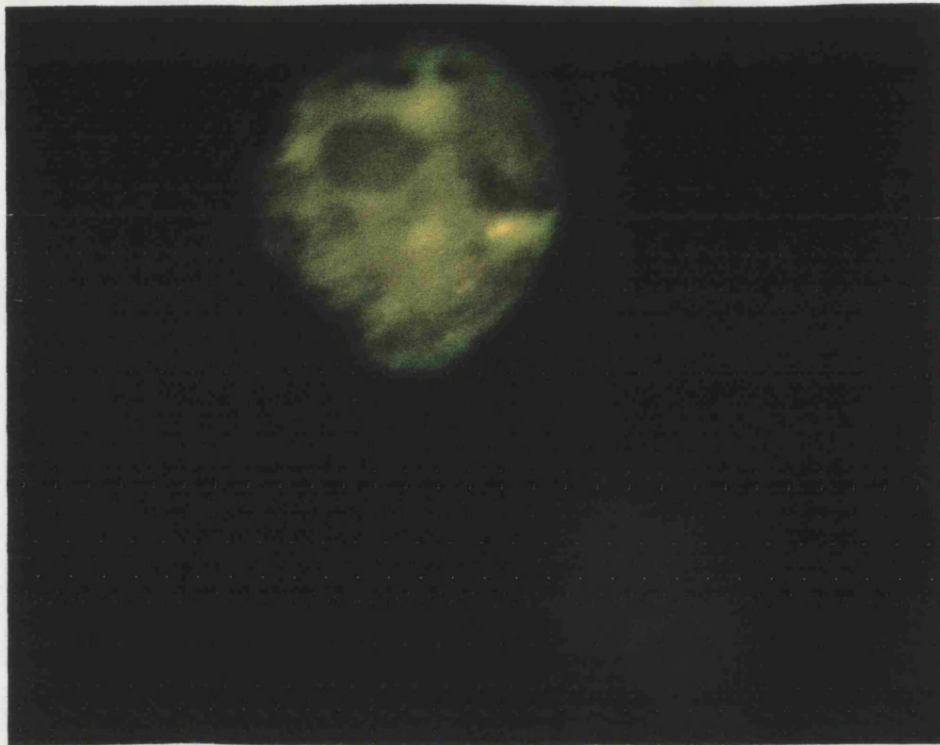
AMs, PBMs and U937 cells were all cultured in complete media on tissue culture petri dishes, 1×10^7 cells per point at a concentration of 1×10^6 per ml. They were cultured with and without inflammatory mediators for periods of up to 24 hours, before removal of the culture supernatant and rinsing with sterile PBS.

2.7.2 RNA extraction

All buffers, chemicals, glassware and disposables used in RNA work were sterile and the RNAses were removed by treatment with Diethylpyrocarbonate (DEPC). Following rinsing the cells were lysed with a guanidinium isothiocyanate buffer (see appendix for all buffers used in northern blotting) as previously described (Jonas *et al.* 1985; Chomczynski and Sacchi, 1987). 2 mls of cold buffer was added to 1×10^7 cells, kept on ice and the cells scraped vigorously with a cell scraper to disrupt the membranes. The

Plate 2.1 RFD immuno-fluorescent staining

RFD1+ AM, with a negatively stained RFD1- AM in the foreground



A mixed cytospin of RFD7+ and RFD7- AMs

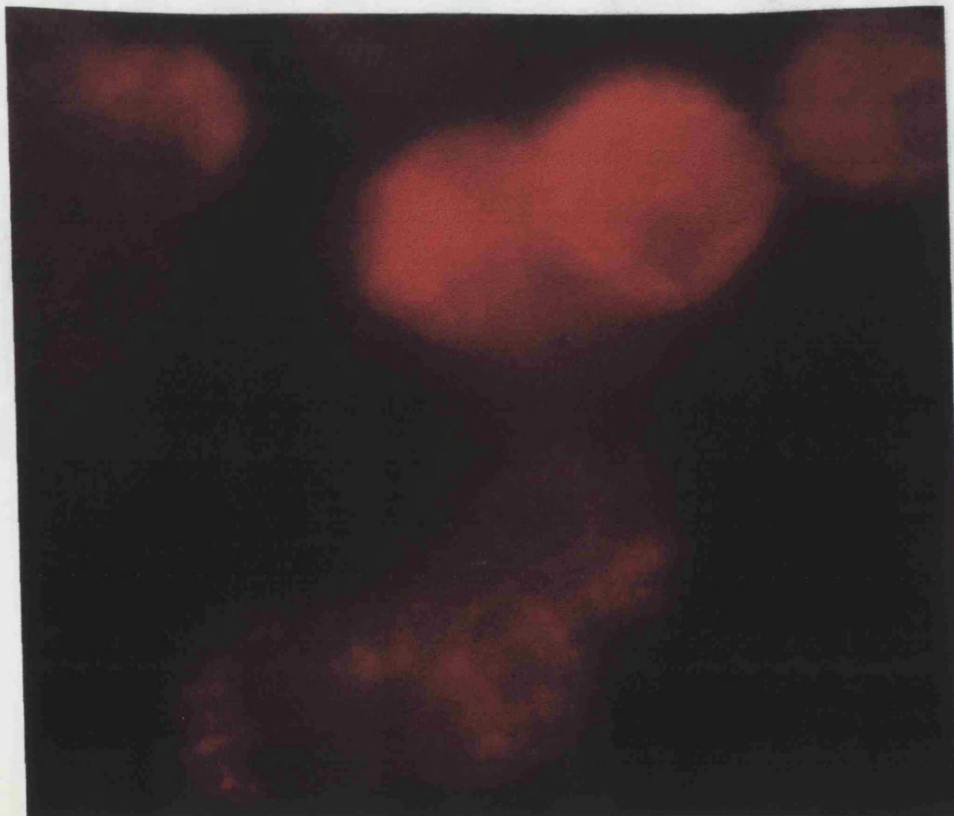
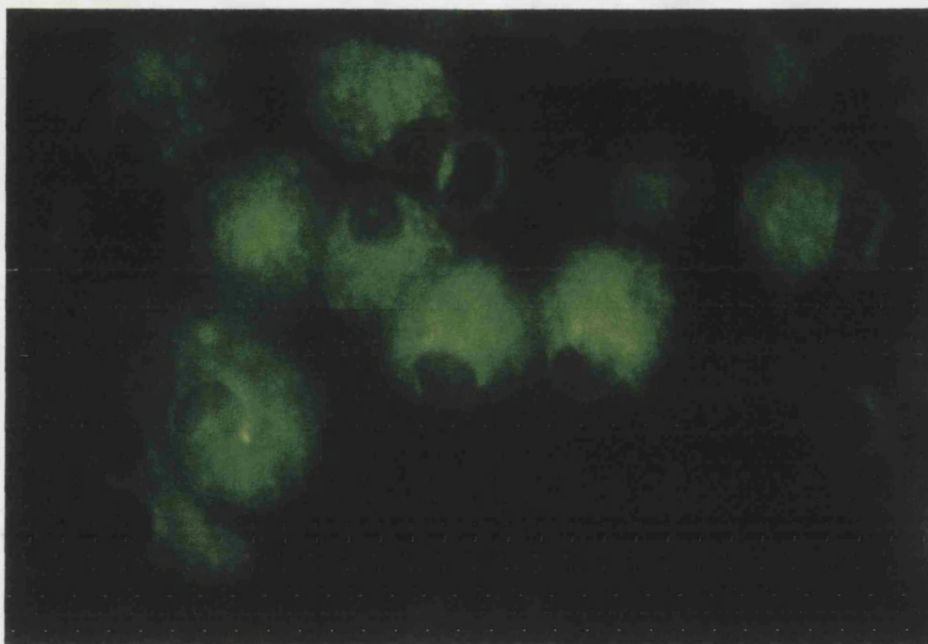
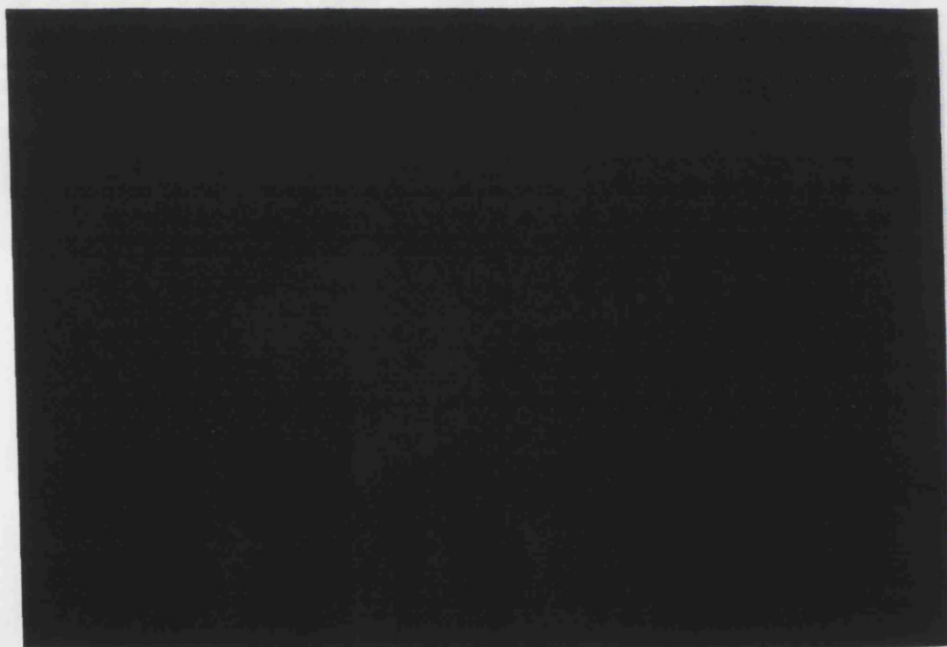


Plate 2.2 CD68 immuno-fluorescent staining



A negative control AM incubated with the corresponding isotype mouse IgG1



lysates were then transferred into 15 ml sterile centrifuge tubes (Falcon) and kept at -70°C overnight to facilitate the lysis process. The following day the lysates were defrosted on ice and the RNA extracted by addition of 1:1 phenol chloroform and phenol extraction buffer. The samples were then centrifuged at 4000 rpm for 10 minutes at 4°C. The aqueous phase containing the RNA was transferred to fresh tubes and the process repeated. Chloroform isoamyl alcohol (24:1) was then added for a further spin at 4000 rpm, 4°C for 7 minutes and the aqueous phase transferred to fresh tubes containing 300 µl of sodium acetate. 3 mls of propan-2-ol was then added and the tubes mixed gently before being placed at -70°C for at least an hour. After defrosting the lysates were centrifuged at 21000 rpm, 4°C in an ultracentrifuge (Beckman) for 1 hour. The supernatants were removed and the RNA precipitates kept in 1 ml of ice cold ethanol overnight at -70°C. The following day the pellets of RNA were washed 3 times in ice-cold ethanol in a microfuge (Jouan) at 4°C and left in a fume hood for an hour to remove all moisture. The pellets were then resuspended in 30 µl of DEPC-treated water. The RNA content was determined by addition of 2 µl of the RNA solution to 980 µl of 0.1M NaOH in a quartz cuvette and measuring the absorbance at 260 nm and 280 nm on a spectrophotometer. 260 nm gave the RNA content and 280 nm gave the protein content, which should never be more than 50% of the RNA value. The RNA concentration was established as follows:

$$A_{260} \times \text{dilution factor} \times 40 = \mu\text{g/ml RNA}$$

The RNA concentration was then corrected to ensure equal loading of 10 µg of RNA per lane.

2.7.3 Gel electrophoresis of RNA samples

Samples were run on 1% agarose gels with formaldehyde which were set in gel trays in the fume hood 1 hour before use. The RNA samples (30 µl volumes) were incubated with 30 µl of sample buffer containing 1 µl of 5 mg/ml ethidium bromide, vortex mixed and incubated on a hot block (Techne) at 80°C for 30 minutes. 2.5 µl of bromophenol blue was then added to each sample and mixed, before loading of the samples on to the gel. The gel was maintained in 3-(N-morpholino) propane sulphonic acid (MOPS) buffer at 4°C in a horizontal sub-cell (Hoefer) and the electrophoresis performed at 100 V for 2.5 hours to separate out the bands of RNA according to size. The gels were then

photographed under UV light on a transilluminator (UVP Products) and the image photographed with a Polaroid to establish equal loading. The gel was then washed in diethyl pyrocarbonate (DEPC) treated water for 30 minutes to remove traces of formaldehyde before transblotting the RNA material overnight on to positively charged nylon membranes (Boehringer Mannheim) using 20x SSC, and paper towels as the wick.

2.7.4 Probing of nylon membranes

Following overnight transfer of RNA the membranes were fixed by baking in an oven at 120°C for 30 minutes. The blots were then pre-hybridised at 42°C with 20 mls of hybridisation solution for an hour before incubation overnight at 42°C with the DIG-labelled oligo probes (R and D Systems) diluted to 10 ng/ml in 2.5 mls of hybridisation solution. The following day the blots were washed at the hybridisation temperature with SSC and 0.1% SDS, before washing for 5 minutes with shaking, at room temperature with a maleic acid (buffer 1 - see appendix) wash buffer. The blots were then blocked for 30 minutes with a combination of maleic acid and blocking stock solution (buffer 2) before a 30 minute incubation with 20 mls of anti-DIG antibody conjugate diluted 1:10000 in buffer 2. The blots were then washed 3 x 10 minutes in wash buffer before addition of 1 ml of lumigen substrate (Boehringer Mannheim) diluted 1:100 in buffer 3. The blots were sealed in a fresh hybridisation bag and incubated in the dark for 30 minutes at 37°C before exposing to x-ray film (Kodak omat) for 1-2 hours in cassettes in the dark. The autorads were then developed immediately in a film developer (Fuji).

2.7.5 Densitometry

Developed autorads and gel Polaroid's were analysed on a calibrated densitometer (GS-670, Bio-Rad). The images were relayed on to a processing package (Molecular Analyst, Bio-Rad) which permitted volumetric analysis of RNA bands. Polaroid photographs of ribosomal RNA were also scanned on the densitometer to determine the quantity of RNA loaded relative to the mRNA of interest, so allowing quantitative evaluation of northern blots.

2.8 Statistical analysis

Data were analysed using the CStat package for Windows on the PC. ELISA data following a normal distribution was analysed using the Student's Paired T Test, whereas non-parametric data was subjected to a Mann Whitney U Test. Wilcoxon Signed Rank was the preferred test for analysis of AM phenotype distribution. All data were expressed as mean \pm SEM. Statistical significance was considered if *p* values were less than 0.05. Statistical evaluation was only performed if sample groups exceeded $n = 3$.

3. TUMOUR NECROSIS FACTOR- α REGULATION BY INTERLEUKIN-10 IN NORMAL SUBJECTS

3.1 Introduction

TNF- α was initially recognised as a cytokine which was capable of inducing vascular necrosis and tumour shrinkage in mice (Carswell *et al.* 1975). It has since become apparent that TNF- α is a highly pleiotropic mediator capable of inducing many beneficial as well as deleterious effects during an inflammatory response, including vascular endothelial cell permeability (Burke-Gaffney and Keenan, 1993; Nawroth and Stern, 1986; Horvath *et al.* 1988), PMN influx and degranulation (Chollet-Martin *et al.* 1992) and fibroblast proliferation (Sugarman *et al.* 1985). TNF- α can also elicit the release of a range of other pro-inflammatory cytokines (Billiau and Vandekerckhove, 1991; Jablons *et al.* 1989; Camussi *et al.* 1991). This wide range of physiological responses indicates that inappropriate regulation of TNF- α could lead to undesirable injurious effects. This is supported by the presence of elevated levels of TNF- α release in pulmonary inflammatory diseases such as sarcoidosis (Foley *et al.* 1992), fibrosing alveolitis (Zhang *et al.* 1993) and ARDS (Tran Van Nhieu *et al.* 1993; Jacobs *et al.* 1989), and amelioration of animal models of lung injury by the use of anti-TNF antibodies (Piguet *et al.* 1989; Tracey *et al.* 1987a). Understanding the regulation of pulmonary TNF- α may give insight into its role in the pathogenesis of lung injury, and has stimulated research into identifying mediators which may fulfill this role.

IL-10 may be one such mediator. A cytokine product of T and B lymphocytes (Yssel *et al.* 1992) as well as PBMs and macrophages (Fiorentino *et al.* 1991; Sieling *et al.* 1993; Howard and O'Garra, 1992), it has known anti-inflammatory properties such as inhibition of antigen presenting cell function (Ding and Shevach, 1992; Villanueva *et al.* 1993) by reduction of B7 (Ding *et al.* 1993a) and class II MHC expression on macrophages (de Waal Malefyt *et al.* 1993), and monocytes (de Waal Malefyt *et al.* 1991b). Formerly known as cytokine synthesis inhibitory factor (CSIF) its modulation of cytokine production has been documented,

showing an ability to inhibit the release of IL-1 (Fiorentino *et al.* 1991; Ralph *et al.* 1992; Muldoon *et al.* 1994), IL-6 (Fiorentino *et al.* 1991) and IL-8 (Cassatella *et al.* 1993) from murine peritoneal macrophages and human blood monocytes. IL-10-mediated inhibition of TNF- α has been demonstrated in macrophage cell lines (Fiorentino *et al.* 1991), mouse peritoneal macrophages (Oswald *et al.* 1992b; Bogdan *et al.* 1992) and PBMs (Ralph *et al.* 1992; Muldoon *et al.* 1994).

In order to determine the possible pathological effects it was first essential to determine the relationship between IL-10 and TNF- α in the normal human lung. This study has investigated the effect of IL-10 on TNF- α mRNA and protein production in human PBMs and AMs, in order to partially characterise the mechanisms of TNF- α inhibition by IL-10 in these cell species. TNF- α protein has been measured by ELISA to establish any effects of IL-10 on protein release. In addition mRNA was isolated from AMs and PBMs to determine any effects IL-10 may have at the level of transcription. The protein synthesis inhibitor cyclohexamide and transcriptional inhibitor actinomycin D were utilised to determine the requirement for *de novo* protein synthesis and any increase in transcript degradation respectively.

3.2 Experimental design

Sample Collection

AMs for this study were obtained from seven non-smoking healthy volunteers (mean age 25.3 years \pm 1.2) consenting to fibreoptic bronchoscopy and bronchoalveolar lavage (BAL) and venepuncture was performed on all bronchoscopy subjects prior to the procedure. Blood was collected into heparinised tubes or blood collection bags (Fenwall, Baxter Healthcare). 50 mls of blood was taken for supernatant analysis, 500 mls for northern analysis. AMs and PBMs were isolated from BAL and peripheral blood respectively to establish any differences in the relationship between IL-10 and TNF- α in the lung and in the periphery.

Cell Culture

AMs and PBMs were cultured for twenty four hours since this was the optimum timepoint for TNF- α protein recovery in the supernatants. Control samples were cultured in medium alone without FCS, although each control was matched with a sample containing medium plus 5 μ g/ml polymyxin B. This compound binds to LPS, and since these samples if contaminated with LPS would have lower detectable cytokine levels than the control this enables detection of contaminated cultures. Such cultures were eliminated from the study. Cells were stimulated with LPS at a dose of 10 μ g/ml - a dose found to be optimum for TNF- α production by AMs. The optimum concentration for PBMs was 100 ng/ml, but it was desirable to have identical stimuli for both cell species since this is a likely scenario *in vivo*.

Protein determination

Recombinant human IL-10 was added at a dose range of 1 - 200 U/ml to the cultures, one hour prior to LPS addition. Spontaneous TNF- α released by AMs and PBMs was low and extrinsic IL-10 had no effect on basal levels (data not shown) therefore only the effects of IL-10 on LPS-induced TNF- α were explored. TNF- α protein was determined by ELISA as described in Chapter 2.

mRNA determination

PBMs were cultured in the presence of 10 μ g/ml LPS alone, and with a dose range of IL-10 (0.1 - 100 U/ml). Control samples contained medium alone. The temporal window for IL-10

inhibition of TNF- α was established by addition of IL-10 at various time points before and after LPS addition. The established optimum for IL-10 addition was found to be 100 U/ml IL-10 added 1 hour prior to LPS and this was selected for the AM experiments. Anti-IL-10 antibody was added at 10 μ g/ml to some AM cultures immediately prior to IL-10 to determine the specificity of the IL-10 response. 5 μ g/ml of the translational inhibitor cyclohexamide (Obrig *et al.* 1971) was administered to some cultures to determine whether *de novo* protein synthesis was required to mediate the effects of IL-10. 5 μ g/ml actinomycin D, a transcriptional inhibitor (Sobell, 1985) was used to arrest all protein synthesis to establish whether the rate of steady state mRNA degradation was altered by IL-10. TNF- α mRNA content was determined by northern analysis as described in Chapter 2. The mRNA content was related to the levels of ribosomal RNA loaded on to the gels. The detection of mRNA from a housekeeping gene such as β -actin would have been more appropriate but methodological difficulties prevented this.

3.3 Results

3.3.1 IL-10 suppresses the production of extracellular TNF- α from LPS-treated AMs and PBMs in a dose and time dependent manner

Constitutively AMs and PBMs produced low levels of TNF- α protein ($0.641 \text{ ng/ml} \pm 0.160$ and $0.058 \text{ ng/ml} \pm 0.018$ respectively) which was elevated to $3.508 \text{ ng/ml} \pm 0.629$ and $2.035 \text{ ng/ml} \pm 0.284$ respectively when maximally stimulated with LPS (Figure 3.1a and b respectively). The LPS-mediated increase in TNF- α protein was significantly abrogated by IL-10 at doses of 50 U/ml and above. 50 U/ml IL-10 reduced AM TNF- α protein by 37% to give $2.035 \text{ ng/ml} \pm 0.284$ ($p < 0.01$) whereas PBMs gave a greater than 60% reduction which translated to $0.698 \text{ ng/ml} \pm 0.167$ ($p < 0.01$). These data demonstrate that IL-10 significantly suppresses LPS-induced extracellular TNF- α from AMs and PBMs.

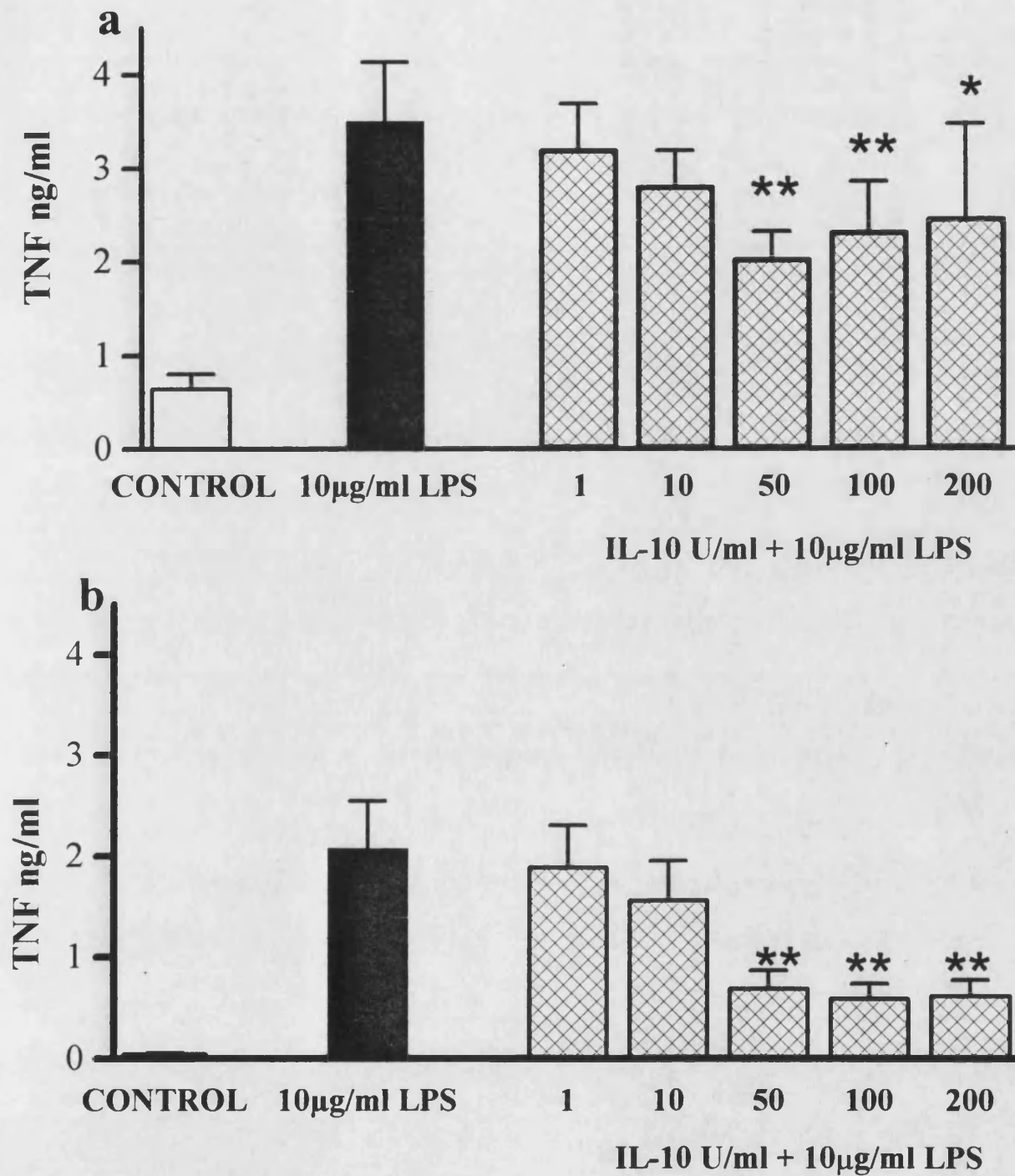
3.3.2 IL-10 inhibits the expression of TNF- α mRNA from LPS-challenged AMs

Having established a decrease in TNF- α extracellular protein secretion by AMs we wanted to assess the effect of IL-10 administration on TNF- α mRNA levels. AMs (10^7 per point) were pre-treated for 2 hours with 100 U/ml IL-10, followed by a further 1 hour incubation with 10 $\mu\text{g/ml}$ LPS. Northern analysis of AM-extracted mRNA revealed a percentage reduction of 47.8 ± 15.23 ($p < 0.05$) in the level of TNF- α message in cultures containing 100 U/ml IL-10 (Figure 3.2) ($n=3$). Addition of 10 $\mu\text{g/ml}$ rat anti-human IL-10 abrogated this response completely and actually increased the effect of LPS alone by $21.06 \pm 9.2 \%$.

3.3.3 Effect of IL-10 pre- and post-treatment on TNF- α mRNA levels from LPS-treated PBMs

To establish the temporal window of IL-10 mediated suppression of TNF- α mRNA, PBMs were treated with 100 U/ml IL-10 at either 1 or 2 hours prior to LPS challenge, simultaneously, or 15 and 30 minutes post-LPS. Total RNA was extracted 1 hour after LPS administration. Pre-treatment with IL-10 ensures substantial suppression of message, as shown in Figure 3.3 (representative of 3 experiments). A reduction in the inhibitory effect of

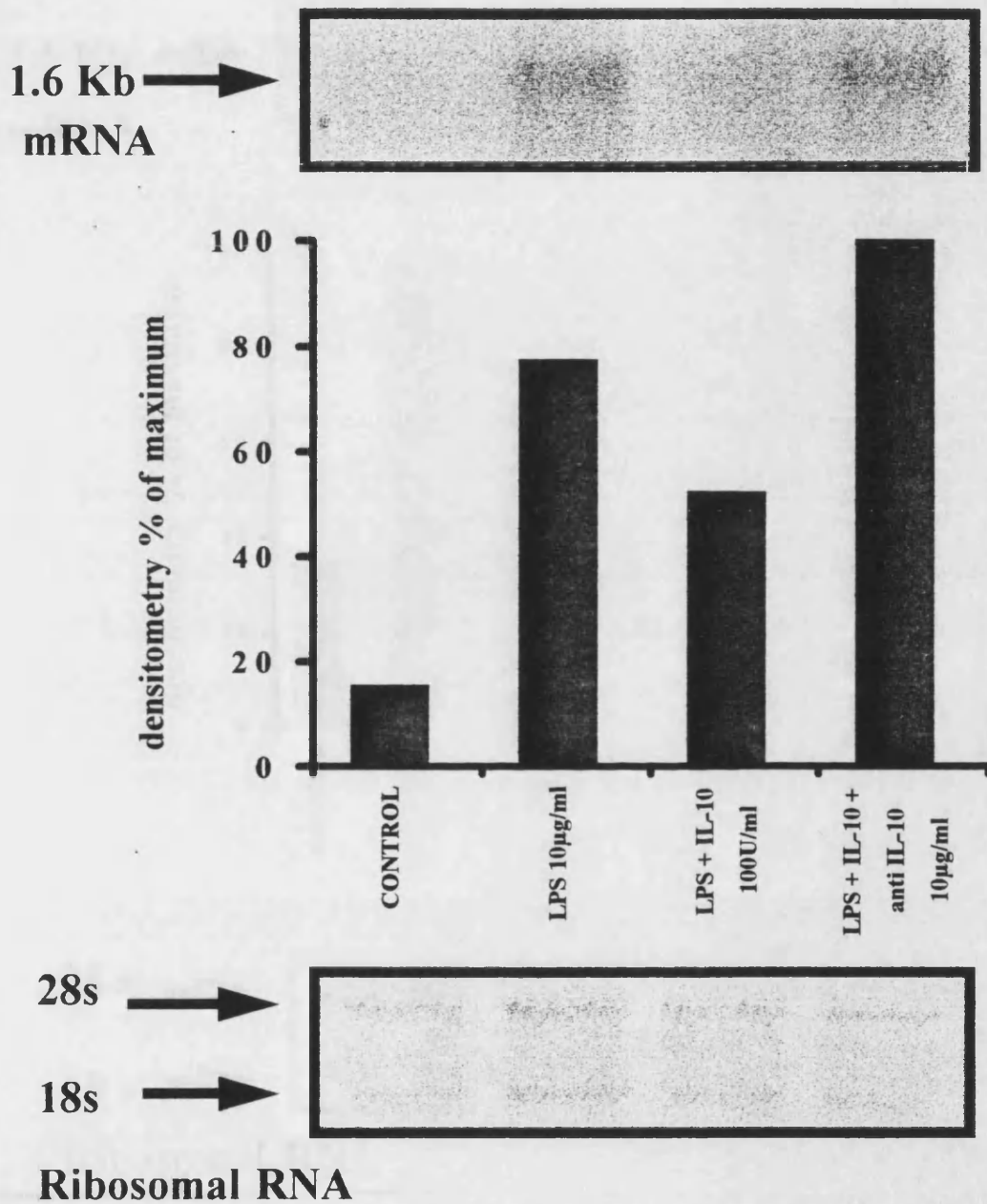
**Figure 3.1 THE EFFECT OF IL-10 ADMINISTRATION
ON LPS-INDUCED TNF- α RELEASE BY AMs and PBMs (n = 6)**



AMs (a) and PBM (b) from healthy subjects, 1×10^6 cells per ml, were cultured for 24 hours with 10 μ g/ml LPS in the presence of absence of a dose range of IL-10. The control contained medium alone.

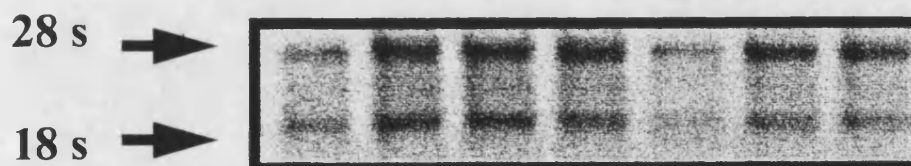
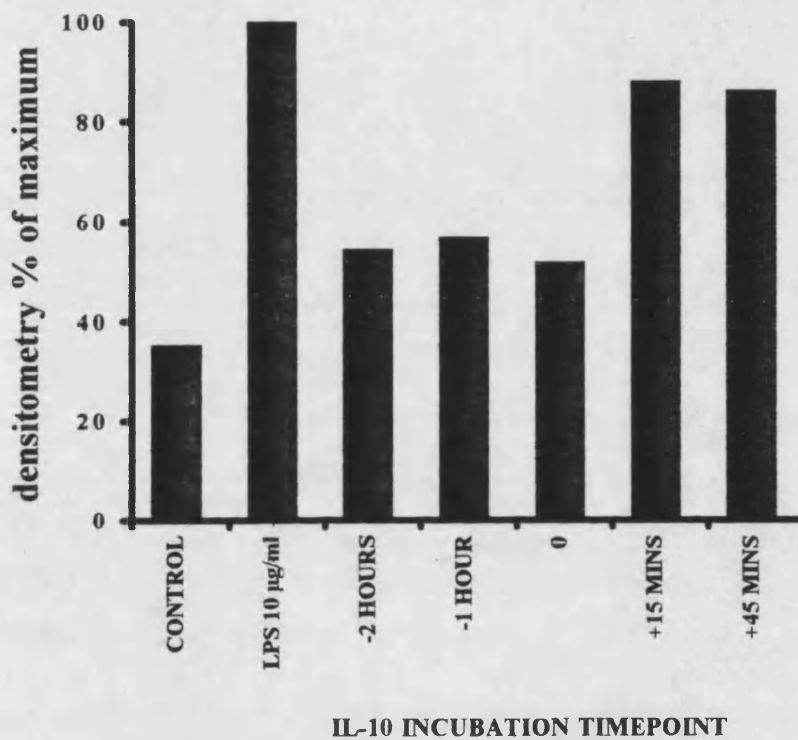
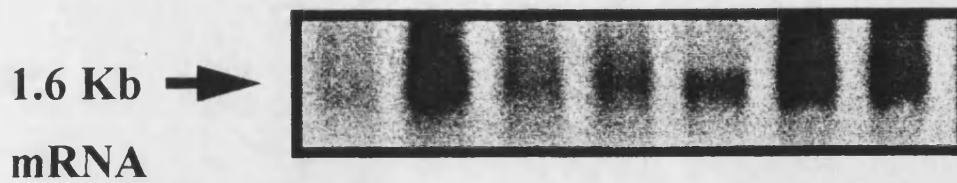
* $p < 0.05$, ** $p < 0.01$ Mann Whitney U test.

**Figure 3.2 IL-10 INHIBITION OF TNF- α EXPRESSION
BY AMs**



Northern analysis of AMs derived from a healthy subject. 10×10^6 cells per point. Control AMs contained medium alone. Cultures containing IL-10 with and without antibody were incubated 1 hour prior to LPS addition. AMs were lysed 1 hour subsequently. Representative of three experiments.

**Figure 3.3 THE TEMPORAL WINDOW FOR THE EFFICACY OF IL-10
CO-ADMINISTRATION WITH LPS**



Ribosomal RNA

Northern analysis of AMs from a healthy subject. 10×10^6 per point. AMs were incubated with 100 U/ml IL-10 before and after 10 µg/ml LPS addition. AMs were lysed 1.5 hours after LPS administration. Representative of three experiments.

IL-10 of 32 % (from 43 to 11% inhibition) was shown at an interval as short as 15 minutes after LPS stimulation.

3.3.4 IL-10 inhibits the expression of TNF- α mRNA from LPS-challenged PBMs

Having established a decrease in TNF- α extracellular protein secretion by PBMs the effect of IL-10 administration on TNF- α mRNA levels was assessed. Cells were pre-treated for 2 hours with IL-10 (dose range 0.1 - 100 U/ml) before addition of 10 μ g/ml LPS. Cells were lysed after a further hour of incubation and northern analysis was performed to determine mRNA levels. The data shows that 100 U/ml IL-10 is the optimum dose for TNF- α mRNA suppression, giving a percentage reduction of 83.1 ± 4.17 ($p < 0.01$) (see Figure 3.4, representative of 6 experiments). No loss of cell viability was observed at all doses of IL-10 used up to 500 U/ml.

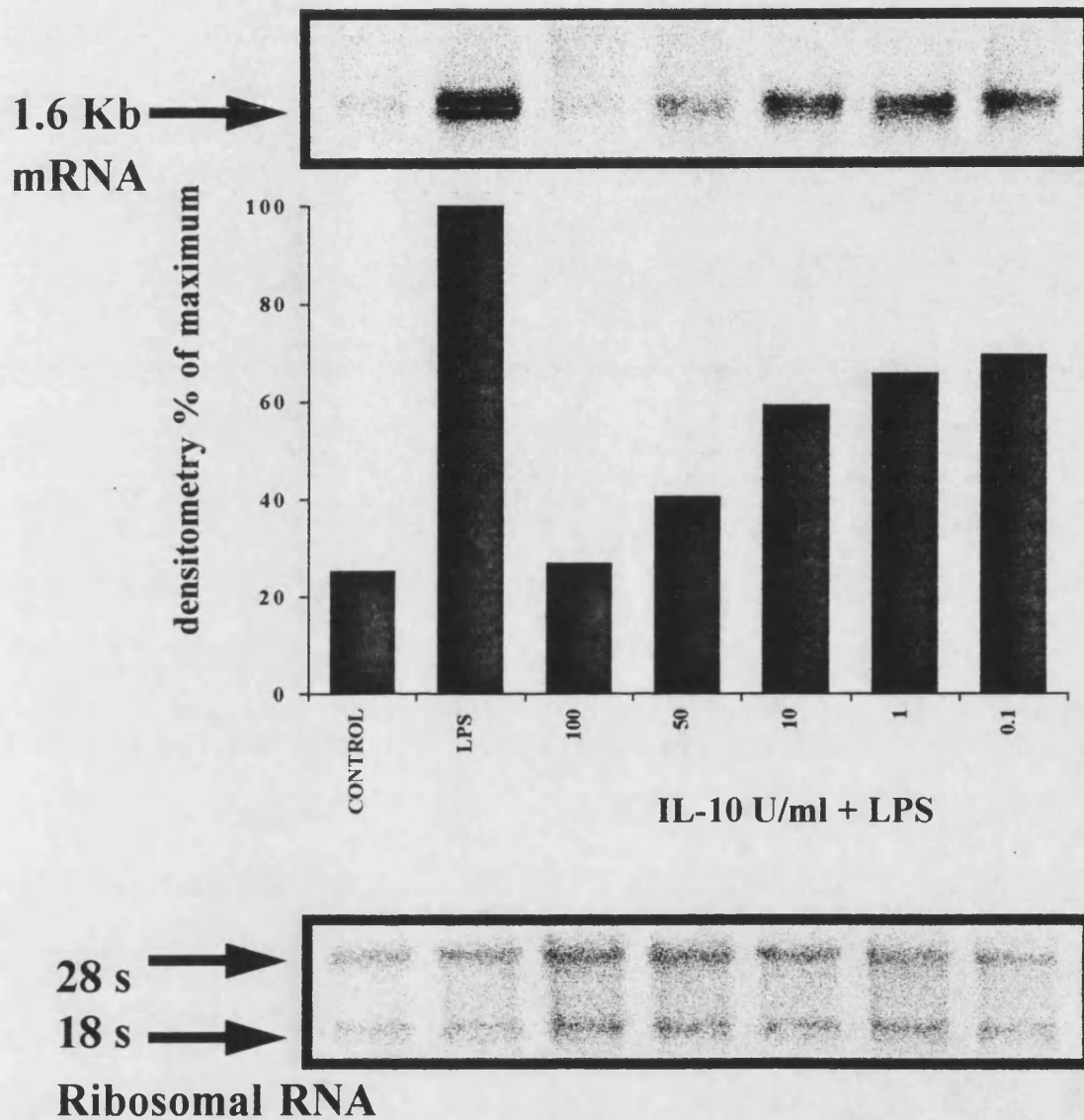
3.3.5 IL-10 induced suppression of TNF- α is not dependent upon *de novo* protein synthesis

To determine if *de novo* protein synthesis was involved in IL-10 induced suppression of TNF- α , PBMs were treated with and without IL-10 plus 5 μ g/ml cyclohexamide 2 hours prior to LPS addition. Cyclohexamide was added 10 minutes prior to IL-10. Total RNA was extracted 1 hour later. TNF- α mRNA with and without LPS treatment was elevated by cyclohexamide treatment (Fig. 3.5), but addition in the presence of 100 U/ml IL-10 led to a decrease in LPS and cyclohexamide-induced TNF- α message by 83.4%. This data, which is representative of 3 experiments indicates that IL-10 can directly suppress the transcription of TNF- α mRNA without the requirement for *de novo* protein synthesis.

3.3.6 The effect of IL-10 administration on the rate of TNF- α mRNA decay

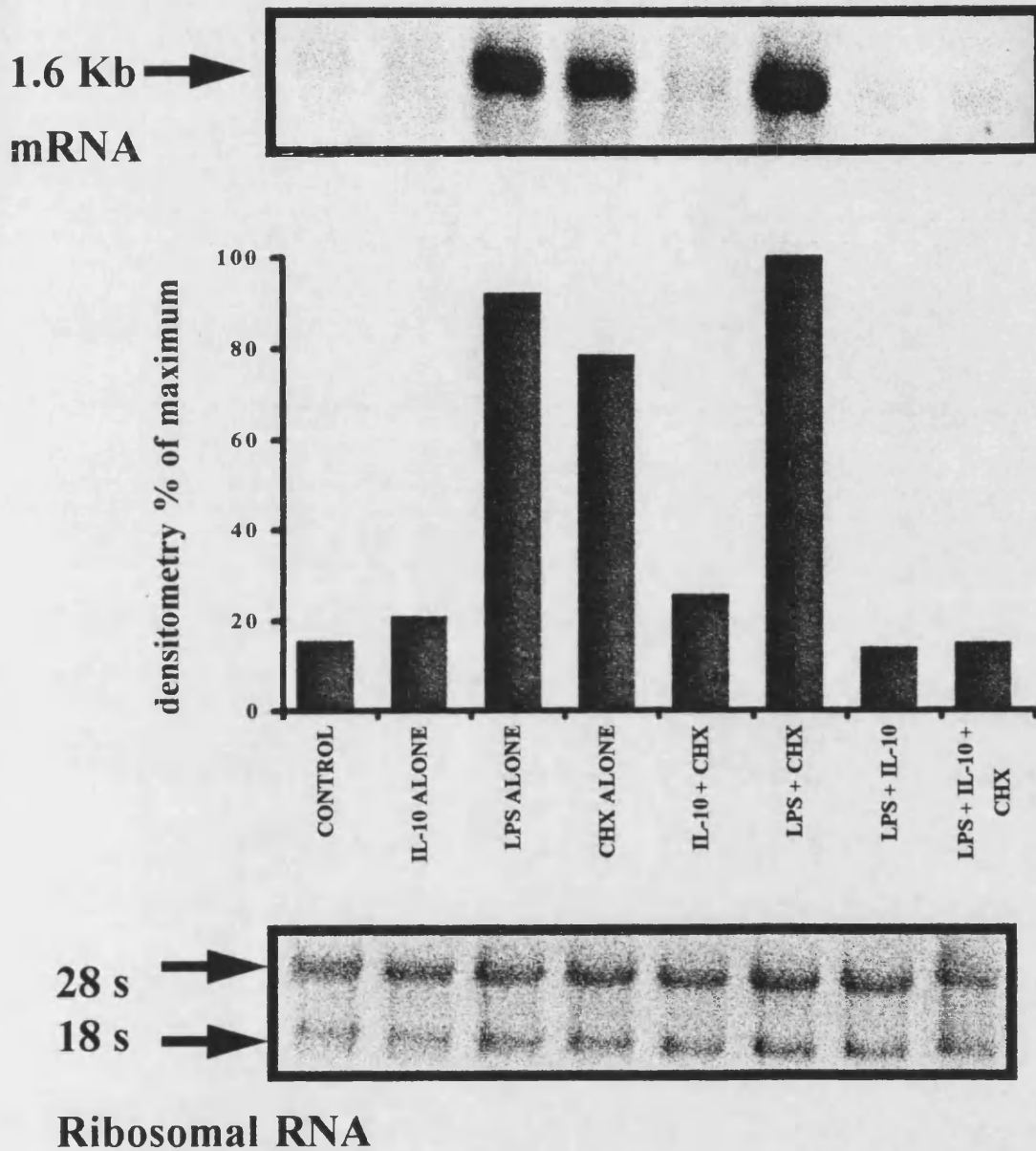
The effect of IL-10 on mRNA half life was assessed by mRNA stability experiments. PBMs were cultured with and without 100 U/ml IL-10 pre-treatment before addition of 10 μ g/ml LPS. Following 1 hour LPS incubation 5 μ g/ml actinomycin D was added, and cells lysed at

**Figure 3.4 THE EFFECT OF A DOSE RANGE OF IL-10
ON TNF- α EXPRESSION IN PBMs**



Northern analysis of AMs from a healthy subject, 10×10^6 cells per point. AMs were pre-incubated for 1 hour with a dose range of IL-10 before LPS addition. AMs were lysed 1 hour subsequently. Representative of six experiments

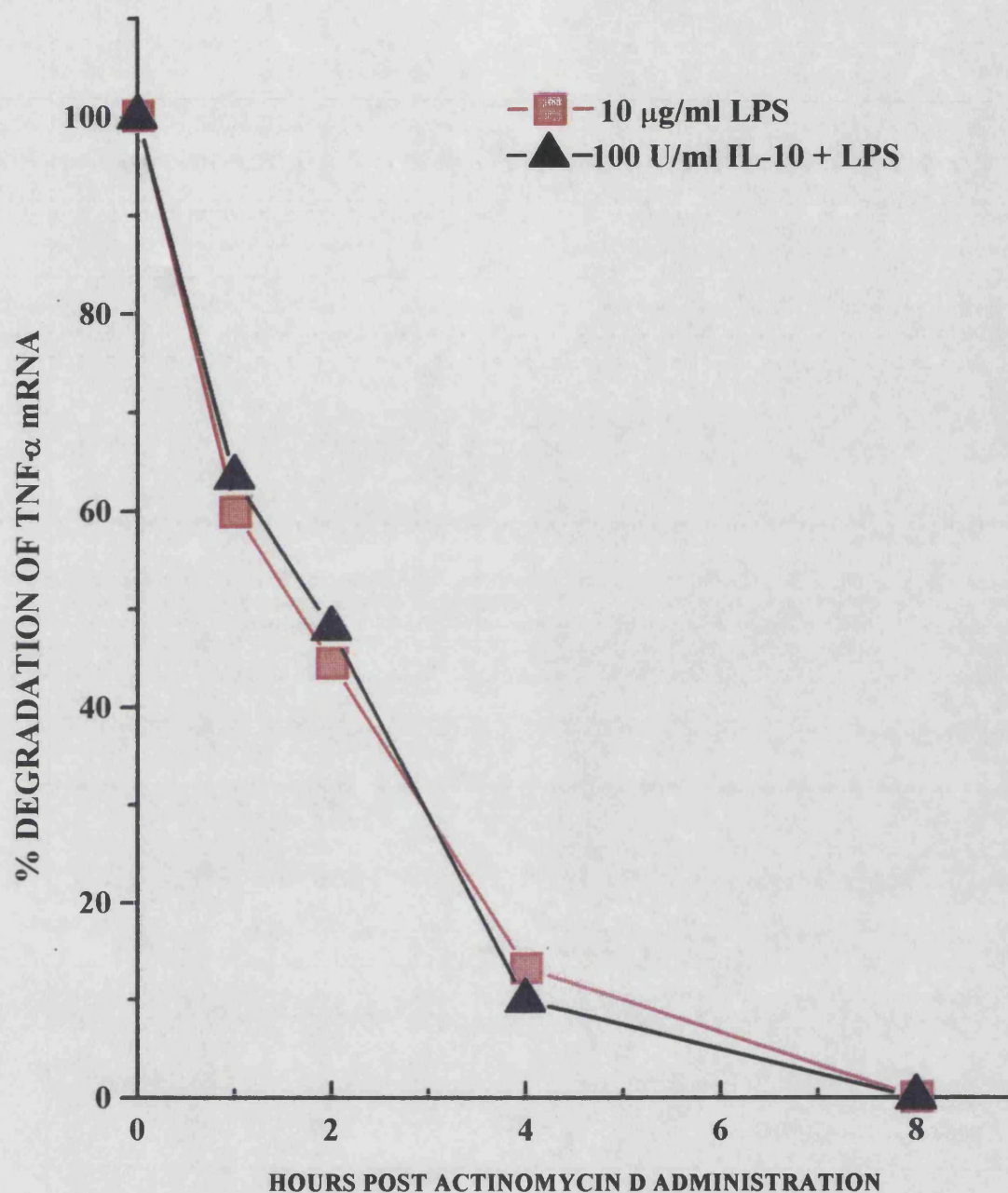
Figure 3.5 THE REQUIREMENT OF *de novo* PROTEIN SYNTHESIS TO MEDiate THE EFFECTS OF IL-10



Northern analysis of AMs from a healthy subject. 10×10^6 cells per point. AMs were pre-incubated with 100 U/ml IL-10 and/or 5 μ g/ml CHX for 1 hour before addition of 10 μ g/ml LPS. AMs were lysed 1 hour subsequent to LPS addition. Representative of three experiments.

1, 2, 4 and 8 hours following actinomycin D addition. No differences were seen in the rate of TNF- α mRNA decay between the IL-10 treated group and the group treated with LPS alone (n=3) (Figure 3.6).

Figure 3.6 mRNA DEGRADATION IN RESPONSE TO IL-10 AND LPS IN PBMs



AMs, 10×10^6 per point, were cultured with 10 µg/ml LPS in the presence of absence of 100 U/ml IL-10 for 1 hour. Actinomycin D, 5 µg/ml was then added to arrest transcription and the degradation of TNF-α mRNA was monitored over an 8 hour period by densitometry.

3.4 Discussion

This study has confirmed that IL-10 is able to down-regulate TNF- α production to some extent in AMs and PBMs in normal subjects, but does not reduce its production to base-line levels. It first needs to be recognised however that this study (and the remainder of this thesis) was an *in vitro* investigation and it may not reflect the circumstances *in vivo*. Cells may be activated by the retrieval process and the involvement of other cytokines and soluble factors in TNF- α regulation by IL-10 cannot be determined by such experiments. However it is still valid to perform *in vitro* studies in order to evaluate the potential relationship between cytokines in a tightly controlled setting. Our findings suggest that both PBMs and AMs are susceptible to inhibition of LPS-induced TNF- α by IL-10, with PBMs perhaps showing the greater sensitivity, as previously reported (Strieter *et al.* 1989a; Martinet *et al.* 1988). In this study AMs produced more TNF- α protein and were less susceptible to the effects of IL-10 than PBMs, suggesting that TNF- α may be the major cytokine for host defence in the AM but not necessarily the PBM. However the role of IL-10 produced by the cells in culture was not taken into account and this would need to be evaluated in future since increased production by AMs *in vitro* could account for the apparent enhanced sensitivity to IL-10. It is also noted that not only could the effects of IL-10 be reversed by addition of anti-IL-10 antibody, but there was also an enhancement of TNF- α production. This may be due to intrinsic production of IL-10 by the AMs which is able to feed back on the cell in an autocrine fashion. Both cell types demonstrate a reduction in LPS-induced TNF- α by IL-10, in terms of released protein and at the mRNA level. This could not be explained by cytotoxic effects of IL-10, since no loss of cell viability was observed. The data presented indicates that the inhibitory effects of IL-10 on PBMs and AMs are seen at the level of TNF- α mRNA expression when stimulated by LPS. Although the direct effects of IL-10 on translation of TNF- α mRNA or the secreted product were not explored, we can say that the reduction in protein release is due, at least in part, to a reduction on TNF- α mRNA expression. This is an important regulatory mechanism in AMs since they contain very little stored TNF- α protein (Kelley, 1990), responding to inflammatory signals with newly synthesised product. Previous studies have examined in some detail the regulation of TNF- α gene transcription and translation (Beutler *et al.* 1986; Beutler and Cerami, 1989; Han *et al.* 1990; Han *et al.* 1991b; Han *et al.* 1991a) and the specific role of IL-10 inhibition (de Waal Malefyt *et al.* 1991a;

Ralph *et al.* 1992; Cassatella *et al.* 1993; Bogdan *et al.* 1992). Since LPS-induced TNF- α was inhibited by IL-10 (IL-10 had no effect on basal levels of TNF- α) an understanding of IL-10 inhibition requires consideration of the mechanisms of LPS induction of TNF- α . Studies which have investigated the mechanisms by which LPS increases TNF- α production (and hence the possible site of IL-10 activity) are conflicting. It is possible that LPS increases TNF- α production by increasing the rate of transcription of the TNF- α gene to mRNA in the cell nucleus, and/or by increasing the efficiency of transcript translation at the ribosome. Two *in vitro* studies have reported an elevation in the transcription rate of the TNF- α gene in response to LPS (Han *et al.* 1990; Han *et al.* 1991b), whereas Bogdan *et al.* (1992) found that transcription rates were unaffected in mouse peritoneal macrophages. By contrast, Han *et al.* (1991b) report that LPS modified TNF- α at both the transcriptional and translational level by regulating the TNF- α gene promoter and the 3' untranslated region respectively, but that the increase in TNF- α protein was largely due to translational derepression (Han *et al.* 1991a).

The inhibitory effect of IL-10 has been shown to be greatly reduced if added more than 2 hours after LPS administration as demonstrated here and elsewhere (Bogdan *et al.* 1992), by which time TNF- α mRNA levels have peaked (Han *et al.* 1991b). This suggests that IL-10 is important at the early stage of TNF- α induction. One possible mechanism of IL-10 activity would be an increase in the rate of transcript degradation. The TNF- α gene has, in common with other cytokines, a 3' untranslated region with a UA rich motif. Such a sequence has been shown to confer instability on the resultant mRNA transcript (Shaw and Kamen, 1986). It has been reported that LPS-treated murine macrophages have increased ribonuclease activity which degrades mRNA containing the UA rich motif (Shaw and Kamen, 1986), and it is possible that IL-10 enhances production of this, or another ribonuclease. However, the mRNA stability experiments in this study showed no increase in mRNA degradation in response to IL-10, which has been also shown in murine peritoneal macrophages (Bogdan *et al.* 1992). This contrasts with a study investigating inhibition of LPS-induced TNF- α in human neutrophils by IL-10 (Cassatella *et al.* 1993), which suggests that enhanced degradation of mRNA did occur. Conflicting results from different cell systems and species suggest that there are cell and species specific mechanisms of TNF- α inhibition by IL-10.

The effects of IL-10 on TNF- α mRNA were not mediated by *de novo* protein synthesis in this instance, and thus the effect is likely to be direct. There has been little investigation into the specific regulation of TNF- α mRNA by IL-10, but one major study utilising murine peritoneal macrophages did find that the IL-10 effects were cyclohexamide sensitive (Bogdan *et al.* 1992). Once again this discrepancy could be explained by differences between the mouse and human species, but there were also differences in methodology. Bogdan *et al.* (1992) isolated TNF- α mRNA after three hours of treatment using 100 ng/ml LPS, as opposed to one hour of treatment with 10 μ g/ml LPS in this study. They also used a dose of 10 μ g/ml cyclohexamide to inhibit protein translation. Thus their cyclohexamide treatment was more robust, and their LPS treatment weaker than that used in this study. This calls into question the selected dose of LPS used in this study. They also looked at events at a later, and possibly more vulnerable point of activation, and should we have examined the effect of IL-10 at multiple time points a more accurate picture may have been achieved. It cannot be easily determined whether their methodology allowed for a more physiological approach but this is a possibility. It needs to be pointed out that the optimum dose for IL-10 of 100 U/ml may not be physiological. In future chapters of this thesis it will be shown that plasma and BALF IL-10 is in the range of 0.2 - 3 ng/ml. 100 U corresponds to 10 ng, so this seems a little high. However doses of this nature may still be considered appropriate since high localised concentrations found *in vivo* cannot be determined by bulk biological fluid sampling. The data presented here appears to suggest that IL-10 must be acting (at least in part) to inhibit gene transcription of the mRNA since this is the only other mechanism whereby the steady state mRNA level of TNF- α may be reduced. One possible mechanism could be inhibition of NF κ B, a transcription factor which mediates LPS-induced cytokine triggering by binding to the cytokine promoter on the DNA and "switching on" cytokine mRNA transcription (Vincenti *et al.* 1992; Ito *et al.* 1994), and this possibility deserves further study.

This study contains preliminary findings which demonstrate that IL-10 can inhibit TNF- α release by AMs and PBMs to an extent from normal subjects, at least in part by inhibiting TNF- α gene transcription. A more thorough understanding of the mechanisms involved could allow comparison with inflammatory diseases where failure of TNF- α regulation is suspected to play a pathological role.

4. TNF- α REGULATION IN SARCOIDOSIS

4.1 Introduction

Sarcoidosis is a disease characterised by a T cell-mediated alveolitis (Campbell *et al.* 1986; Crystal *et al.* 1981b) which is largely CD4+ in nature (Semenzato, 1986; Hunninghake and Crystal, 1981). This results in the formation of the non-caseating granuloma which are a major contributory factor in the alveolar structural derangement and compromised lung function which may occur in some cases (Crystal *et al.* 1981b). The maintenance of the chronic inflammatory events in sarcoidosis can potentially lead to end-stage fibrosis contributing to the 5% mortality rate associated with the disease (Crystal *et al.* 1981b).

The initial insult which leads to the development of sarcoidosis remains unknown. There has been much debate as to whether the stimulus is environmental or an intrinsic abnormality of the inflammatory response (Walters and du Bois 1995). It has been proposed that infectious agents such as viruses or mycoplasma may be implicated, since high serum antibodies to these organisms have been detected in sarcoidosis patients (Semenzato, 1986). However, it has been noted that there are elevated levels of serum immunoglobulins and circulating immune complexes in patients with sarcoidosis (Semenzato, 1986; Popp and Wachtler, 1991), and there is also an association with the expression of certain antigens in the MHC locus (Semenzato, 1986), all of which are indicative of an autoimmune disease process. It seems possible therefore that the disease may be a manifestation of an individuals abnormal or uncontrolled inflammatory response to a common insult, which in normal circumstances would not lead to a chronic inflammatory event (Walters and du Bois 1995).

An understanding of the mechanisms which lead to the formation and maintenance of the chronic inflammation in sarcoidosis may give insight into aspects of immune regulation. It is known that the disease is characterised by a T cell infiltrate into the alveolar space (Hunninghake and Crystal, 1981; Semenzato, 1986) and that these lymphocytes are in a

more primed state in active sarcoidosis (Crystal *et al.* 1981b; Costabel *et al.* 1985). It is believed that these T cells are drawn into the lung by release of chemokines, possibly from the AM (Folkard *et al.* 1995). The AM is known to be present in elevated numbers in sarcoidosis (Semenzato, 1986) and it also has enhanced capacity to produce cytokines such as TNF- α (Dalhoff *et al.* 1993; Muller-Quernheim *et al.* 1992; Steffen *et al.* 1993), MIP-1- α (Elias, 1988) and possibly IL-1- β (Hunninghake, 1984; Steffen *et al.* 1993; Yamaguchi *et al.* 1988). It is also a more potent antigen presenting cell in the sarcoidosis lung (Lem *et al.* 1985; Gant *et al.* 1991) and possesses a number of monocyte markers such as OKM1 (Hance *et al.* 1985) which suggests that many sarcoid AMs may have recently been drawn into the lung from the circulating monocyte pool (Hance *et al.* 1985; Crystal *et al.* 1981b). This enhancement has lead to the hypothesis that AMs may be involved in the generation and maintenance of the pulmonary inflammatory response and that, together with the T cells are important in the maintenance of the sarcoid granuloma.

A number of studies have addressed the role of the AM in sarcoidosis, and paid particular attention to its capacity to release TNF- α . TNF- α has been found to be elevated in the BALF of sarcoidosis patients and AMs have demonstrated enhanced capacity to produce this cytokine in some studies (Dalhoff *et al.* 1993; Steffen *et al.* 1993; Foley *et al.* 1992; Terao *et al.* 1993; Muller-Quernheim *et al.* 1992), but others have shown little or no increase in TNF- α production in sarcoidosis (see discussion) (Bachwich *et al.* 1986b; Foley *et al.* 1990). In healthy subjects it is assumed that the cytokine network is tightly regulated to prevent pathological consequences. Inhibitory cytokines such as IL-10, IL-4 and IL-13 (Fiorentino *et al.* 1991; Essner *et al.* 1989; Yanagawa *et al.* 1995) have come to prominence as mediators which may have a regulatory role, especially with regard to TNF- α . Circulating inhibitors such as the soluble TNF receptors (TNF-R) and IL-1 receptor antagonist (IL-1ra) are also thought to be important in the control of the cytokine network. Indeed, it has previously been demonstrated that the ratio of IL-1 to IL-1ra in sarcoidosis is balanced in favour of IL-1 (Rolfe *et al.* 1993; Kline *et al.* 1993), so that even if the IL-1 levels are not significantly elevated in the disease, the proportion of bioactive to inactive IL-1 is greater.

This study is based upon the hypothesis that production and/or activity of TNF- α is excessive in the lungs of sarcoidosis patients and this may be due to (1) enhanced capacity of AMs to produce TNF- α , (2) decreased production and or activity of IL-10 and/or (3) decreased production of TNF-R. Spontaneous and LPS-induced production of TNF- α by AMs and PBMs in sarcoidosis has been measured, together with its modification by IL-10, in comparison to normal subjects. In addition, spontaneous and LPS-induced production of IL-10 itself has been measured to establish a possible autocrine relationship with TNF- α . Finally spontaneous and LPS-induced production of the type I soluble TNF receptor (TNF-R) was determined to see how it relates to TNF- α production.

4.2 Experimental Design

AMs and PBMs were isolated from patients with sarcoidosis as described previously (Chapter 2). Patients in this study were undergoing fibreoptic bronchoscopy for diagnostic purposes and were not receiving steroid treatment prior to the bronchoscopy. Patients studied had bilateral hilar lymphadenopathy with minimal pulmonary physiological impairment, with evidence of erythema nodosum and arthralgia. Serum angiotensin converting enzyme (ACE) was 34.17 ± 13.14 (n = 17) (normal range 23 - 85). These patients are classified as having stage one sarcoidosis (Studdy *et. al.* 1990). Patients with stage two and three radiological findings (with evidence of pulmonary infiltrates and interstitial shadowing) were excluded. Diagnosis of sarcoidosis was confirmed by transbronchial biopsy revealing granuloma formation in all patients studied.

Purified AMs and PBMs were cultured at a density of 1×10^6 cells per ml in culture medium. Control wells contained medium alone. To eliminate the possibility of endotoxin contamination affecting results, all cell cultures had additional control wells containing 5 µg/ml polymyxin B sulphate (Sigma) which binds to LPS. Cultures which showed a reduced TNF- α response in the presence of polymyxin were considered contaminated and therefore not considered for the study.

LPS was used as an immune response stimulus in the cultures. Dose response curves were utilised to ascertain sensitivity to LPS, but 10 µg/ml LPS was shown to be the optimum concentration for AMs and was used as the preferred dose when only one concentration of LPS was appropriate. 10 µg/ml LPS was also selected for PBM cultures, despite an optimum dose of 100 ng/ml LPS, so that both cell species would have identical culture conditions. IL-10 was used at a dose range 0.0001 - 100 U/ml in isolation and also in conjunction with 10 µg/ml LPS. Supernatants from the cultures were removed after 24 hours and frozen at -70°C until required. Plasma and cell-free BALF were removed from whole blood and total lavage fluid respectively by centrifugation and stored likewise until required. Lavage protein levels were determined as grammes of protein per million recovered BAL cells. Repeated freeze-thaw cycles were avoided to reduce loss of protein activity.

4.3 Results

4.3.1 TNF- α in plasma and BALF

Plasma and BALF samples were assayed for TNF- α using a double-ligand ELISA. Plasma and BALF from normal volunteers contained no detectable TNF- α protein, in contrast to sarcoidosis plasma and BALF, which contained 0.281 ± 0.084 ng/ml and 0.427 ± 0.077 ng of TNF- α /million BAL cells respectively (Figure 4.1). These values indicate significant elevations ($p < 0.05$ and $p < 0.01$ respectively) in circulating and pulmonary TNF- α in sarcoidosis patients.

4.3.2 TNF- α release by AMs and PBMs spontaneously, and in response to LPS

AMs derived from sarcoidosis patients produced slightly lower levels of TNF- α than normal subjects both spontaneously and in response to LPS, with 10 μ g/ml LPS yielding only 1.46 ± 1.45 ng/ml in sarcoidosis compared with a value of 3.487 ± 0.927 ng/ml from normal AMs (Figure 4.2a). This was not reflected in PBMs, where sarcoidosis and normal subjects gave very similar TNF- α profiles in response to LPS, although spontaneous TNF- α release by PBMs was elevated at 0.91 ± 0.75 ng/ml compared to 0.044 ± 0.008 ng/ml TNF- α in normal PBMs. (Figure 4.2b).

4.3.3 Modification of TNF- α release by IL-10

IL-10 did not significantly reduce the TNF- α protein release by AMs at any dose in sarcoidosis, although it approached significance at 10 and 100 U/ml ($p = 0.074$). This is in contrast to normal AMs, where 100 U/ml gave a significant abrogation ($p < 0.01$) (Figure 4.3a). The lower doses of IL-10 were effective at reducing TNF- α release by sarcoidosis AMs, however, since 0.1 U/ml showed a reduction in this instance whereas normal AMs did not respond to doses of IL-10 lower than 10 U/ml. The PBMs of sarcoidosis and normal subjects gave a similar profile, and both reduced LPS-induced TNF- α optimally at 100 U/ml IL-10 ($p < 0.05$ and $p < 0.01$ respectively) (Figure 4.3b).

**Figure 4.1 TNF- α DETECTED IN THE PLASMA (n = 4)
AND BALF (n = 8) OF SARCOIDOSIS PATIENTS**

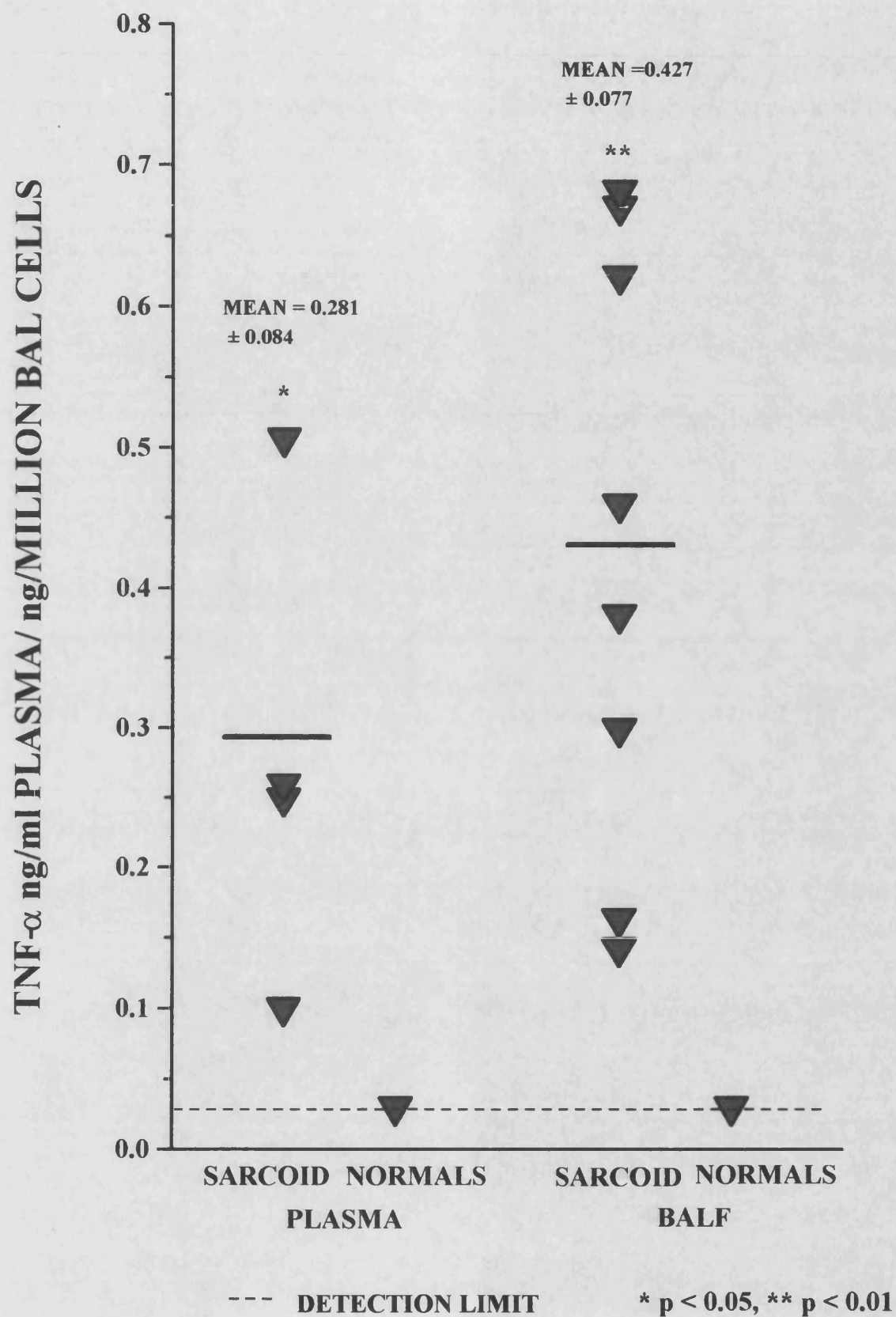
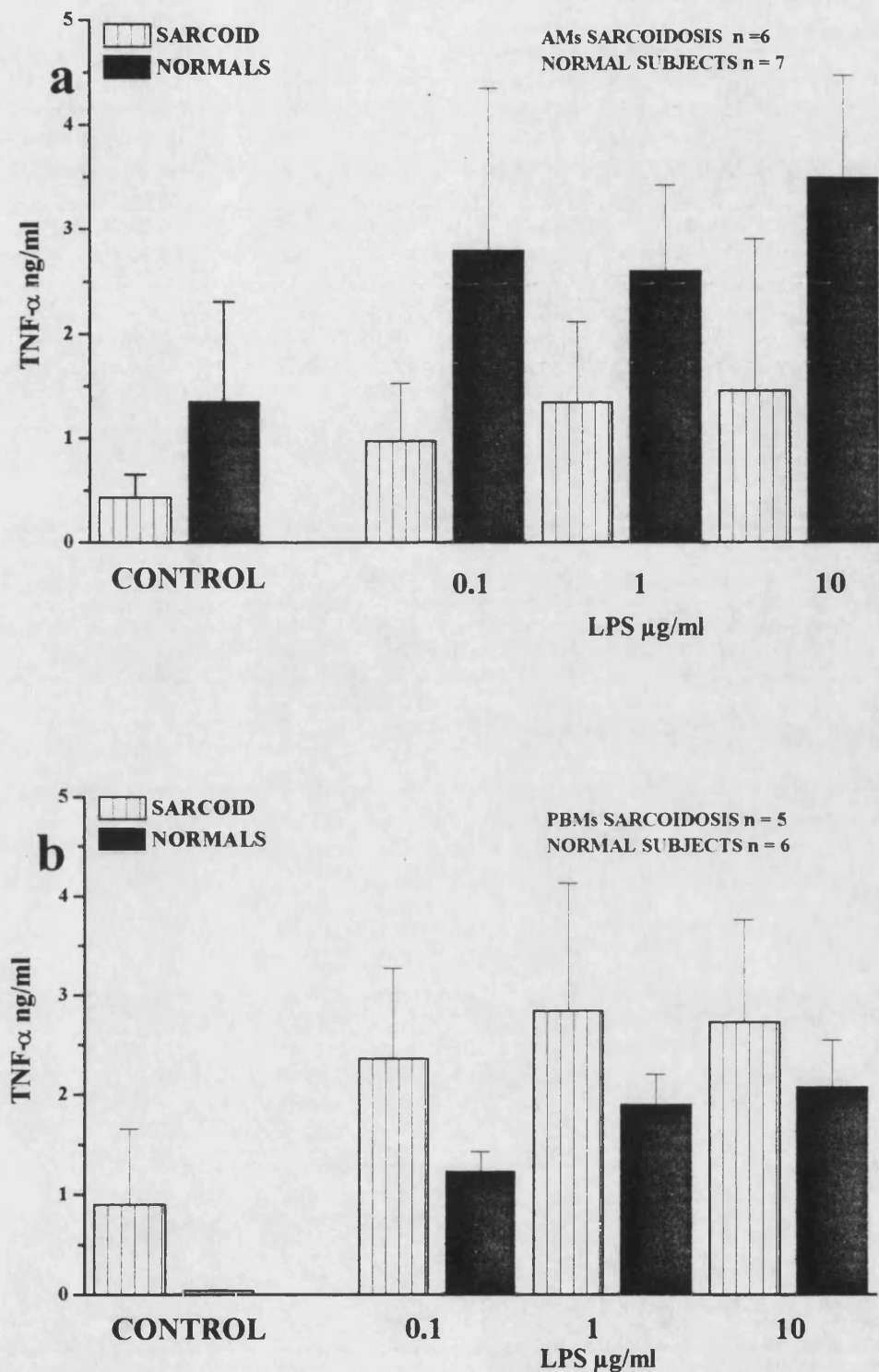
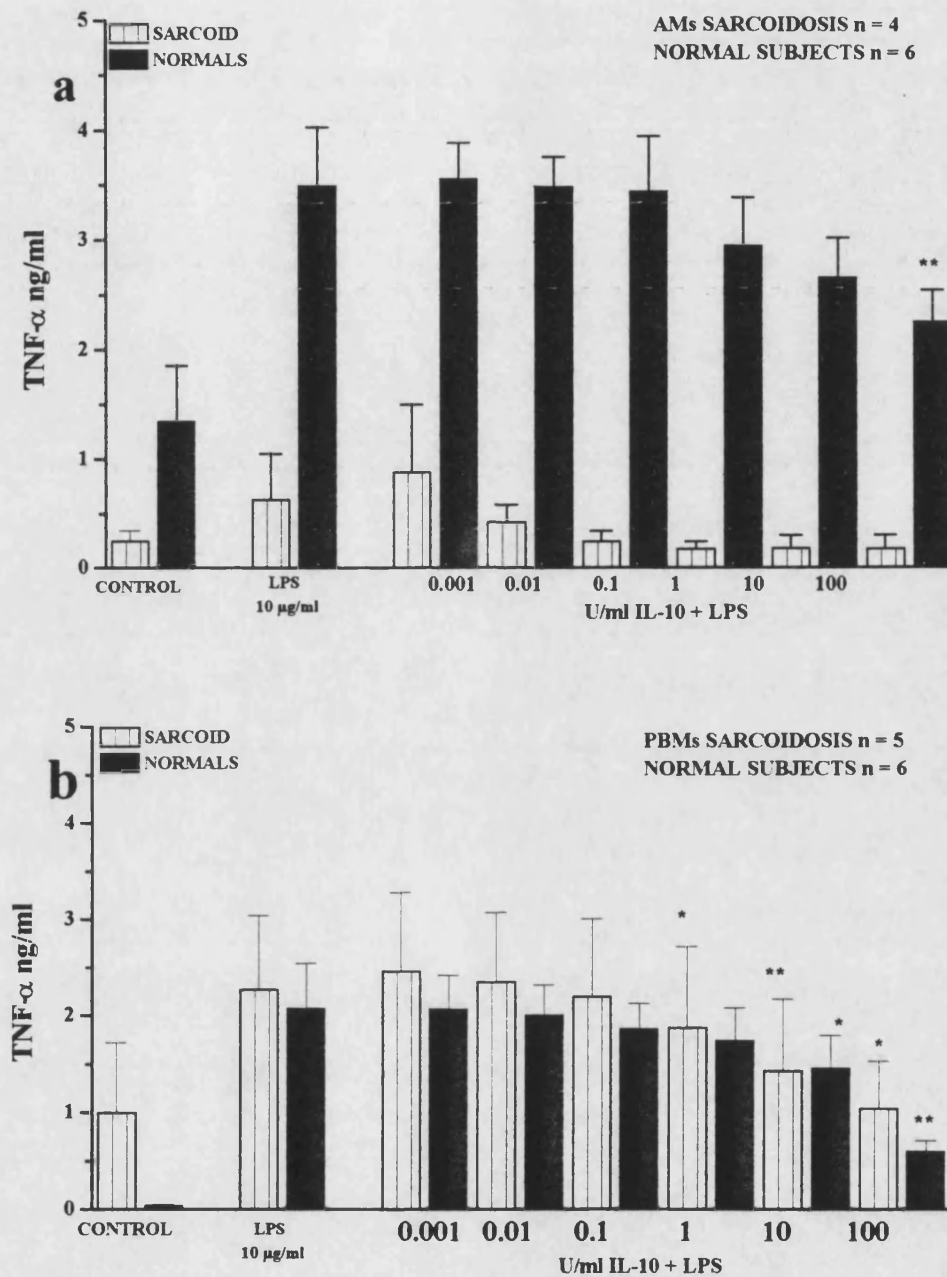


Figure 4.2 THE EFFECT OF LPS ON TNF- α RELEASE BY AMs AND PBMs DERIVED FROM PATIENTS WITH SARCOIDOSIS



AMs (a) and PBMs (b), 1×10^6 cells per ml, were cultured for 24 hours with a dose range of LPS. Control cells were cultured with medium alone.

Figure 4.3 THE EFFECT OF IL-10 ON TNF- α RELEASE BY AMs AND PBMs DERIVED FROM PATIENTS WITH SARCOIDOSIS



AMs (a) and PBMs (b) were cultured for 24 hours, 1×10^6 per ml with 10 μ g/ml LPS in the presence or absence of a dose range of IL-10. Control cells were cultured in medium alone.

* $p < 0.05$, ** $p < 0.01$ Mann Whitney U test

4.3.4 IL-10 levels in plasma and BALF

Low levels of IL-10 were detected in the plasma and BALF of both normal volunteers and sarcoidosis patients (Figure 4.4). They compared very closely, with plasma levels of normal subjects and sarcoids 65.47 ± 28.26 pg/ml and 58.18 ± 31.12 pg/ml respectively and BALF levels 334.5 ± 198.16 pg and 406.47 ± 132.79 pg of IL-10 per million BAL cells respectively. There were no significant differences between the groups.

4.3.5 IL-10 release by AMs and PBMs, spontaneously and in response to LPS

Spontaneous release of IL-10 from sarcoid AMs was greater than normal AMs ($p = 0.053$), with a value of 22.54 ± 9.26 pg/ml from sarcoidosis AMs compared to 11.34 ± 4.62 pg/ml IL-10 from normal subjects (Figure 4.5a). IL-10 from sarcoidosis AMs was elevated by LPS at 0.1 and 1 μ g/ml ($p = 0.053$), whereas normal AMs produced elevated IL-10 only in response to 10 μ g/ml LPS. Interestingly, PBMs from sarcoidosis subjects produced apparently much higher levels of IL-10 than normal PBMs, both spontaneously and in response to LPS, but this was not significant due to very high standard errors (not shown), (Figure 4.5b).

4.3.6 TNF-R levels in plasma and BALF

TNF-R was significantly elevated in the plasma of sarcoidosis patients compared to normal subjects; 1.644 ± 0.161 ng/ml and 0.421 ± 0.055 ng/ml respectively ($P < 0.001$) (Figure 4.6). TNF-R was also increased in the BALF, where sarcoidosis patients gave 1.482 ± 0.359 ng of TNF-R per million BAL cells compared to 0.254 ± 0.063 ng of TNF-R per million BAL cells for normal BALF ($p < 0.05$).

**Figure 4.4 IL-10 DETECTED IN THE PLASMA (n = 11) AND BALF (n = 19)
OF SARCOIDOSIS PATIENTS**

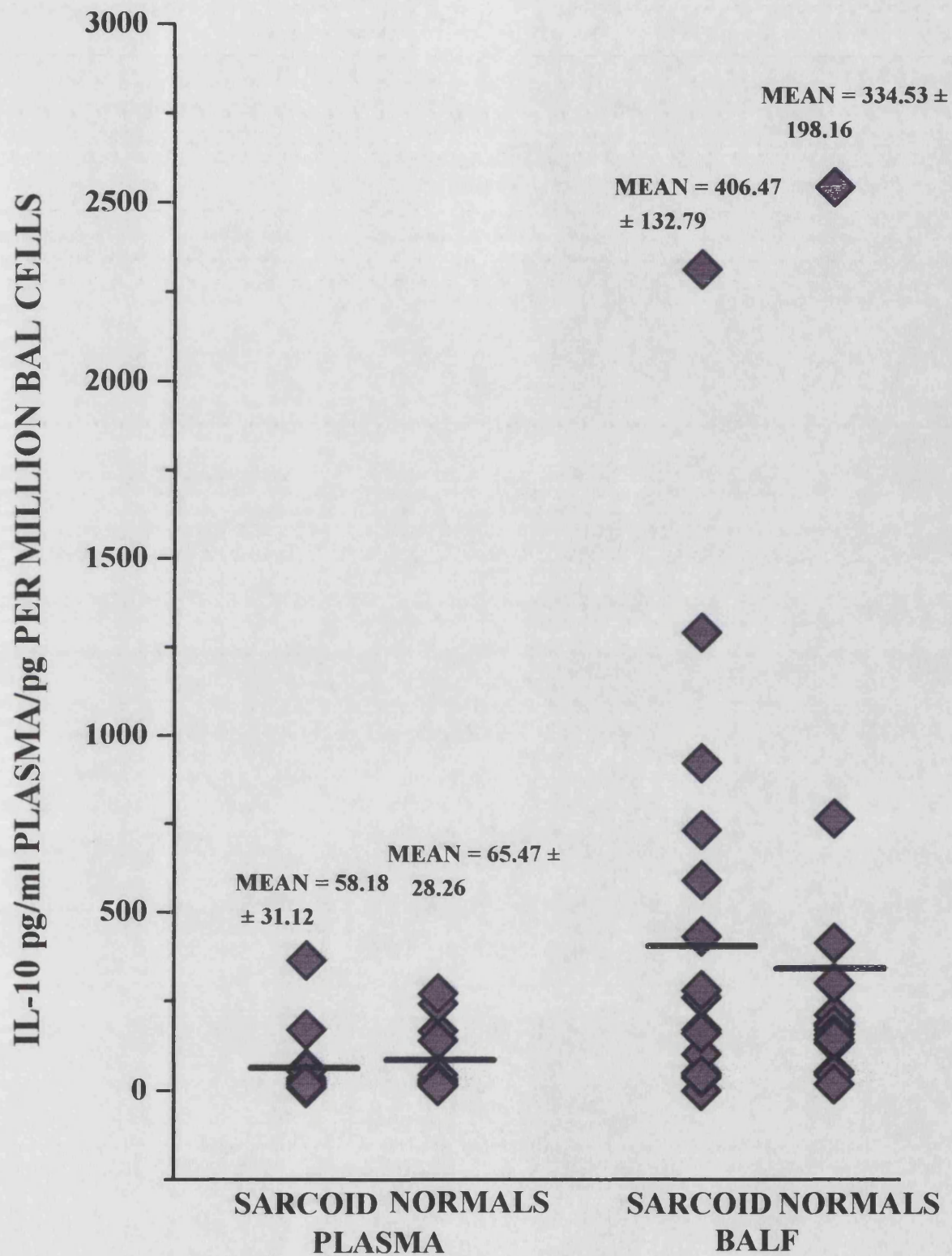
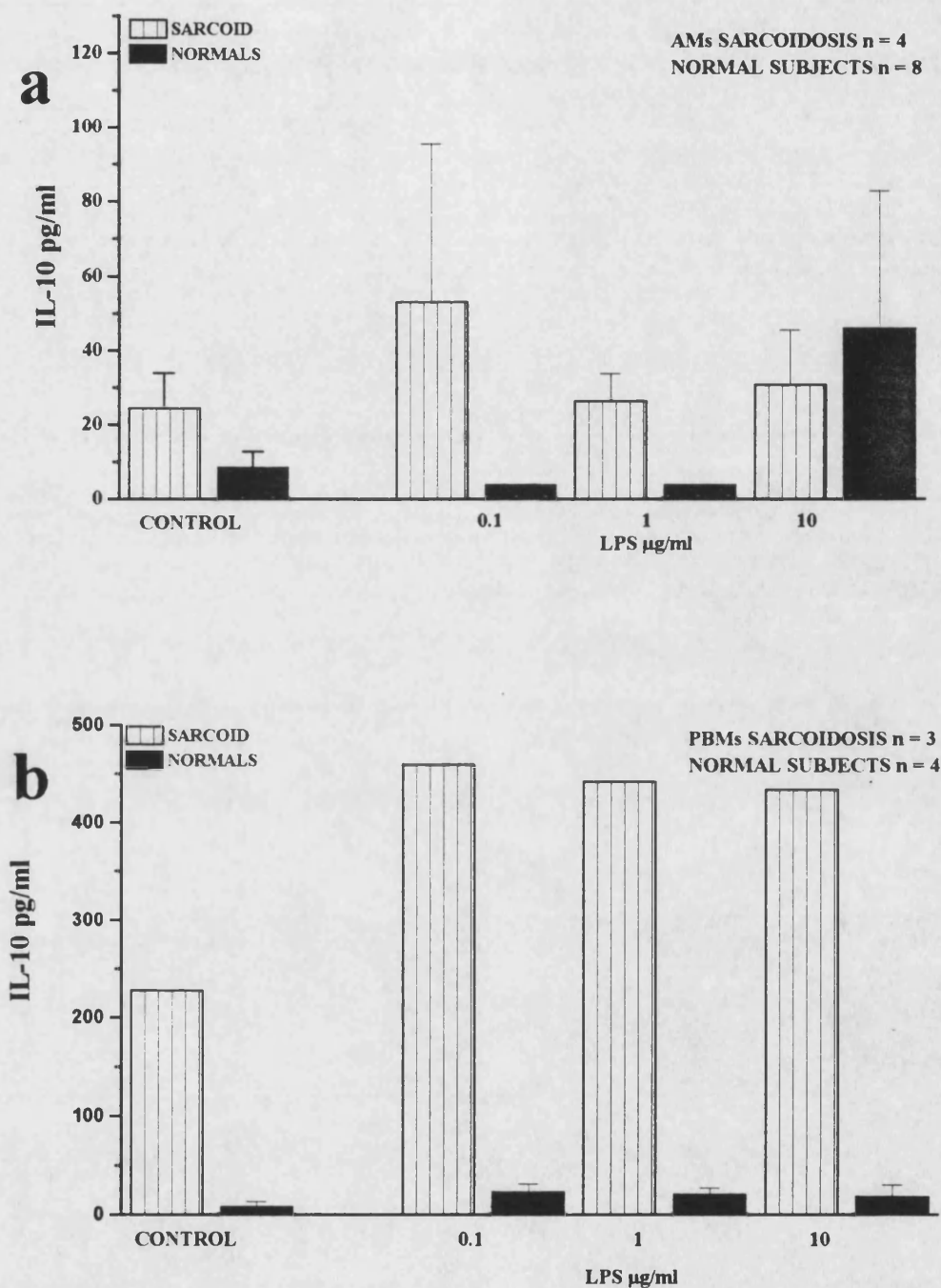
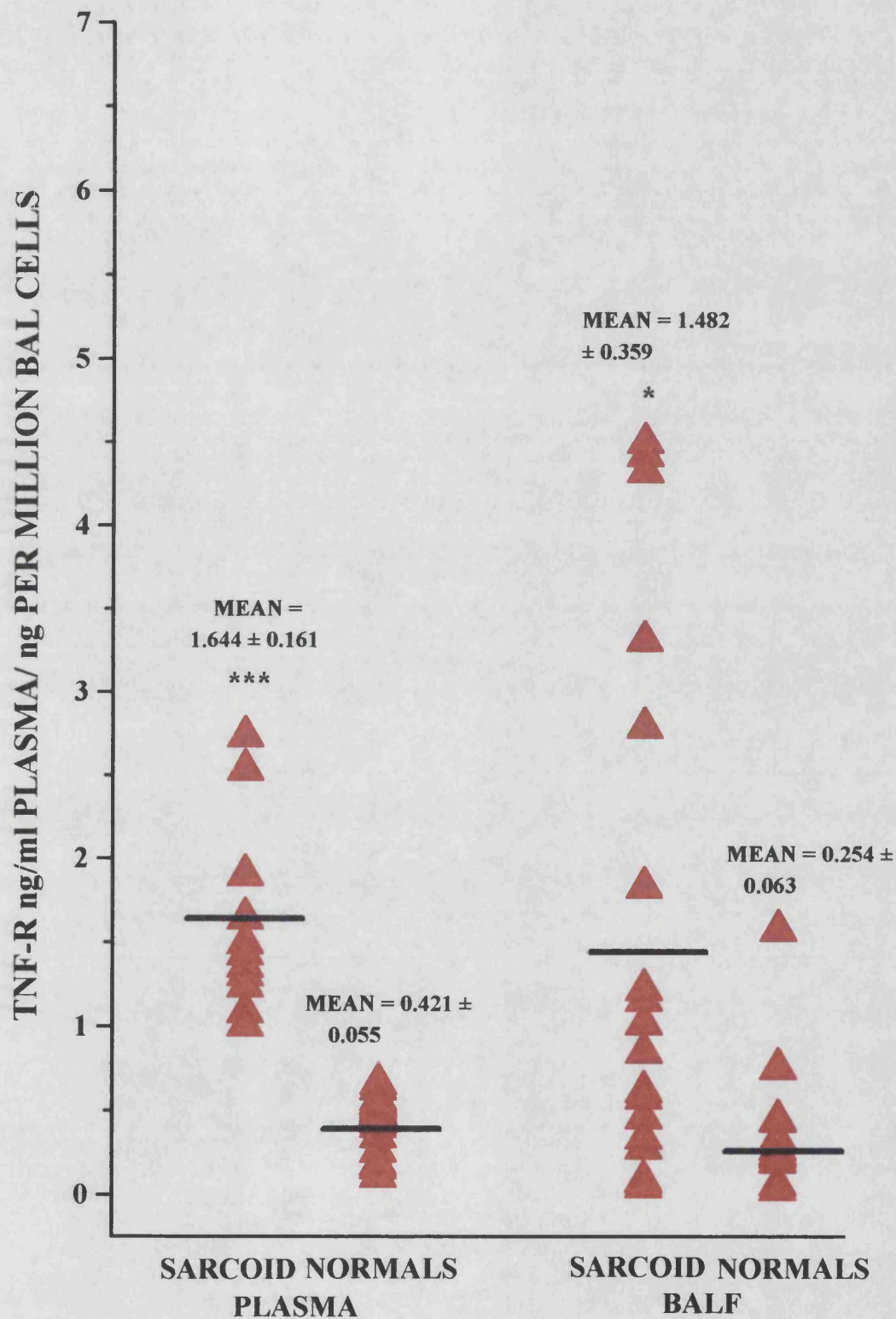


Figure 4.5 THE EFFECT OF LPS ON IL-10 RELEASE BY AMs AND PBMs DERIVED FROM PATIENTS WITH SARCOIDOSIS



AMs (a) and PBMs (b) were cultured for 24 hours, 1×10^6 per ml in the presence of a dose range of LPS. Control cells were cultured in medium alone

Figure 4.6 TNF-R DETECTED IN THE PLASMA (n=19) AND BALF (n=11) OF SARCOIDOSIS PATIENTS



** p < 0.05, *** p < 0.001 Mann Whitney U test*

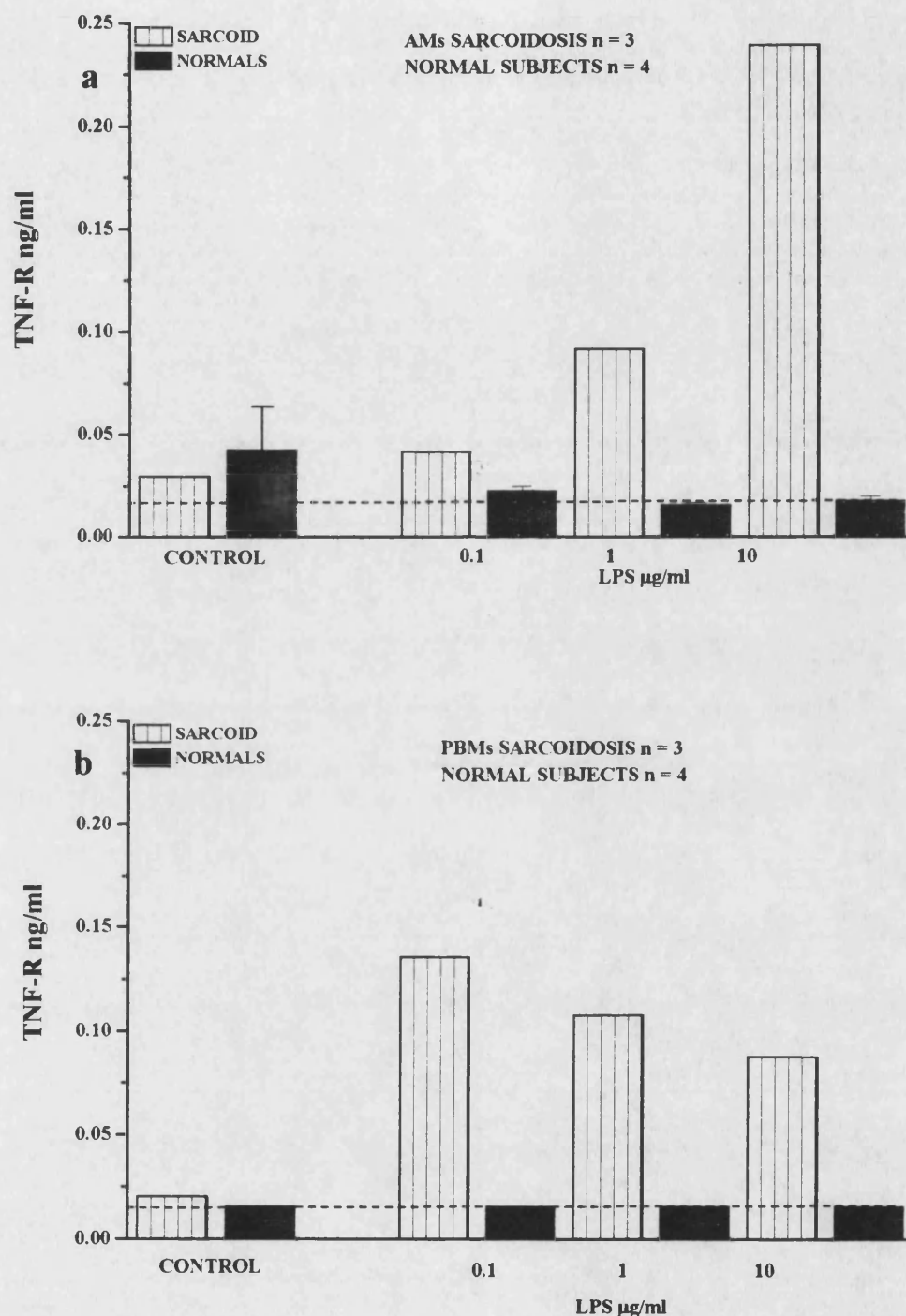
4.3.7 TNF-R release by AMs and PBMs, spontaneously and in response to LPS

Normal AMs produced a spontaneous level of TNF-R (0.044 ± 0.018 ng/ml) which was reduced in a dose-dependent manner by LPS (Figure 4.7a). Sarcoidosis AMs produced a similar level of spontaneous TNF-R production (0.028 ng/ml) which was elevated by LPS at all doses. Production of TNF-R by normal PBMs was below detectable limits, whereas sarcoidosis PBMs produced elevated spontaneous levels which were increased by LPS administration (Figure 4.7b).

4.3.8 Modification of TNF-R release by IL-10

IL-10 alone at all doses increased TNF-R release from sarcoidosis AMs whereas IL-10 in co-culture with LPS resulted in an almost total inhibition of TNF-R (Figure 4.8a). Normal AMs did not produce TNF-R in response to LPS. Sarcoidosis PBMs had their LPS-induced TNF-R levels returned to basal levels by IL-10 at 1 and 10 U/ml (Figure 4.8b). All TNF-R levels from normal PBMs were below detectable limits.

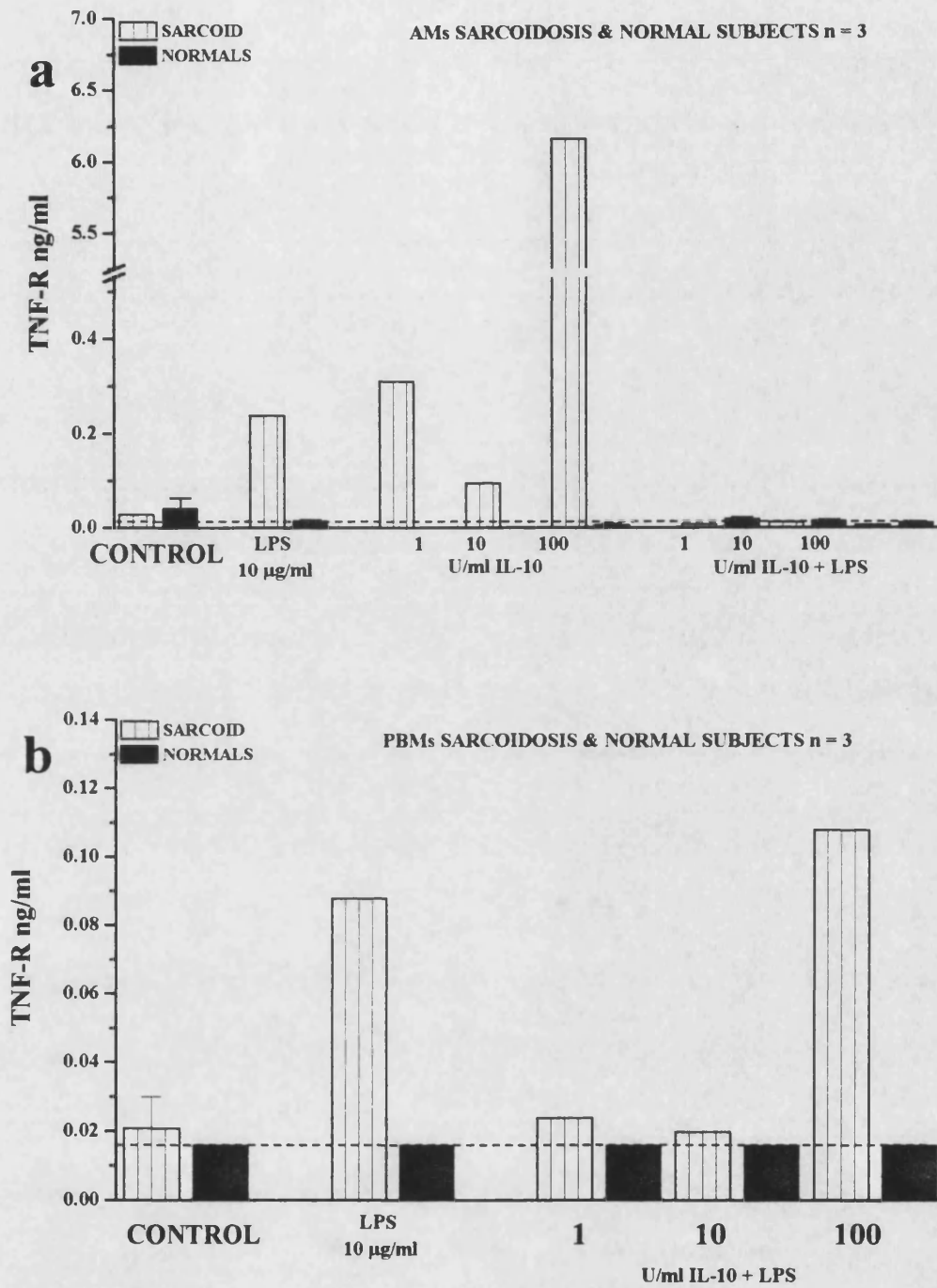
Figure 4.7 THE EFFECT OF LPS ON TNF-R RELEASE BY LPS IN AMs AND PBMs DERIVED FROM SARCOIDOSIS PATIENTS



AMs (a) and PBMs (b) were cultured for 24 hours, 1×10^6 cells per ml, in the presence of a dose range of LPS. Control cells were cultured in medium alone.

----- Detection limit = 0.016 ng/ml TNF-R

Figure 4.8 THE EFFECT OF IL-10 ON TNF-R RELEASE BY AMs AND PBMs DERIVED FROM PATIENTS WITH SARCOIDOSIS



AMs (a) and PBMs (b) were cultured for 24 hours, 1×10^6 cells per ml, plus or minus LPS $10 \mu\text{g/ml}$ and/or a dose range of IL-10. Control cells were cultured in medium alone.

- - - detection limit $0.016 \text{ ng/ml TNF-R}$

4.4 Discussion

The data for sarcoidosis patients overall gives a picture of altered cytokine profiles in the AMs, whereas the PBMs, with the exception of TNF-R follow a pattern which does not differ from the normal picture. This is suggestive of a localised pulmonary inflammatory event with minimal peripheral involvement, and is supported by published works which suggest that PBMs in sarcoidosis are unexceptional (Spatafora *et al.* 1989). Indeed, Muller-Quernheim *et al.* (1992) have shown elevated levels of TNF- α produced by sarcoidosis AMs, but not PBMs. This supports the theory that pulmonary sarcoidosis, despite its systemic nature, may be a compartmentalised disease with localised pulmonary activation, and immune suppression in the periphery. There is evidence of elevated TNF- α and TNF-R in the plasma of patients with sarcoidosis, but with reference to the former this may not necessarily be derived from circulating PBMs, but derived from pulmonary TNF- α which has entered the circulation from the alveolar space or other cells in the circulation.

The TNF- α release demonstrated from sarcoidosis AMs was unexpected since there is no significant difference when compared normal subjects. The majority of studies indicate enhanced production of TNF- α by AMs in sarcoidosis (Dalhoff *et al.* 1993; Steffen *et al.* 1993; Terao *et al.* 1993; Foley *et al.* 1992; Muller-Quernheim *et al.* 1992) although this has not been a consistent finding (Bachwich *et al.* 1986b). There could be several explanations for this discrepancy. Firstly, it may be an indication of AM damage in the sarcoidosis cultures. It has become apparent during this study that AMs derived from sarcoidosis patients are less resistant to the purification process employed here than equivalent cells from normal volunteers and patients with ARDS or CFA. All cells used in this study had their viability confirmed before culture, and sometimes after culture, but this would give no indication as to whether the AMs were damaged but not to the point of cell death. Other studies showing high TNF- α values used apparently identical purification protocols (Dalhoff *et al.* 1993; Steffen *et al.* 1993), suggesting that a methodological explanation is unlikely. AMs from sarcoidosis patients were isolated on the same day as CFA AMs in some instances, with identical protocols and whereas CFA AMs produced expectedly high TNF- α , this was not reflected by sarcoidosis AM cultures. Assuming that the AMs in the study were robust, it is necessary to seek an

alternative explanation for the findings. One possible explanation for the discrepancy is the selection of patients for this study. Patients were included if they fulfilled the criteria of stage one sarcoidosis, which is an acute presentation with a high prospect of complete resolution within two years (Studdy *et al.* 1990). Stages two and three, which demonstrate pulmonary infiltrates are more likely to progress to chronic disease and it is possible that a study involving patients of this type would have been more appropriate for the examination of TNF- α regulation. Indeed some reports of elevated TNF- α included patients with stage two and three in their studies (Foley *et al.* 1992; Terao *et al.* 1993). There was also the lack of distinction in this study between active and inactive sarcoidosis patients, on the basis of CD4:CD8 T cell ratio. A previous study determined that AMs from sarcoidosis patients with active disease produced elevated TNF- α , whereas AMs from patients with inactive disease produced normal levels (Muller-Quernheim *et al.* 1992). We did encounter high TNF- α levels in 2 of our patients who may well have been the only patients in our study with aggressive disease, but this is unconfirmed.

Another likely explanation for the findings could be an inability to detect elevated TNF- α release by AMs by the detection assay due to interference from biological inhibitors of TNF- α . Much of the published data regarding elevated TNF- α used assays which determined total TNF- α , which may or may not be bound to inhibitors and is therefore inactive (Steffen *et al.* 1993; Spatafora *et al.* 1989). This ELISA measures only free TNF- α , so preventing us from detecting TNF- α bound up with TNF-R. It has been observed by one study that sarcoidosis AMs and AMs from patients with tuberculosis (another granulomatous disorder) produce comparable amounts of TNF- α , but the systemic effects of TNF- α are more pronounced in TB, where symptoms such as tissue necrosis and cachexia are commonplace (Rook *et al.* 1987). These manifestations are not apparent in sarcoidosis which suggests that the TNF- α released by sarcoidosis AMs may undergo extracellular modification which would enhance degradation, be exposed to inhibitory cytokines which prevent its actions upon other cells, or be bound up by soluble receptors. Markedly elevated TNF-R was detected in the BALF and plasma of sarcoidosis patients which indicates that BAL cells and/or blood cells are shedding more TNF-R which can then inactivate the TNF- α present. This data demonstrates that the

sarcoidosis AMs although not producing excessive amounts of TNF-R spontaneously may produce more TNF-R in response to LPS, so it is possible that autocrine TNF-R could be interfering with our TNF- α measurements. It should also be considered that the AMs are not the only source of TNF-R in the lung - endothelial cells, neutrophils and T cells all have TNF receptors which may be shed (Burke-Gaffney and Keenan, 1993; Porteu and Nathan, 1990; Tartaglia *et al.* 1993). Also, PBMs in sarcoidosis produce significant amounts of TNF-R both spontaneously and in response to LPS, whereas normal PBMs produced no detectable TNF-R. This puts forward the possibility that *in vivo* the TNF-R may enter the lung from the pulmonary circulation. This study has not addressed the role of the type II TNF receptor, since at the time of investigation there was no available antibody to measure the trace amounts released into cell supernatants, and it is usually produced in much lower quantities (Chikanza *et al.* 1993). However, it has been reported that the type II receptor is the dominant form shed by cells of the myeloid lineage, which includes macrophages (Joyce *et al.* 1995), so it cannot be excluded that the type II receptor may be released in large quantities from AMs, thus preventing detection of TNF- α in our assay. These data suggest that the levels of cytokine may be only half the story, and as shown in studies on IL-1 and IL-1ra (Kline *et al.* 1993), it is the balance of cytokine to inhibitor which determines the potency of the mediator.

A further possible explanation for the low levels of TNF- α released by sarcoidosis AMs in this study is the potential autocrine role of IL-10. Although IL-10 was not found to be elevated in plasma and BALF, sarcoidosis AMs did produce more spontaneous IL-10 than normal subjects. This released IL-10 may feed back on the AM so blocking production of subsequent TNF- α . Wanidworanun *et al* (1993) have suggested an autocrine role for IL-10 in human PBMs, but found that IL-10 was released subsequent to TNF- α so it would be unable to prevent initial TNF- α release. Unfortunately there have been no studies addressing the timecourse of IL-10 production in normal AMs, let alone sarcoidosis AMs so this autocrine role remains to be established. The dose-responsive effects of IL-10 on LPS-induced TNF- α indicate that sarcoidosis AMs may be sensitive to IL-10 doses at least 100-fold lower than normal AMs (in sarcoidosis LPS-induced TNF- α is reduced by 0.1 U/ml IL-10, whereas no reduction is seen in

normal AMs below 10 U/ml). Therefore it is likely to be a potent autocrine effect of IL-10 in sarcoidosis, even when IL-10 levels are comparatively low.

The relationship between IL-10 and TNF-R is complex. IL-10 alone appeared to increase TNF-R release when compared to the basal and LPS response in sarcoidosis AMs. There was no response to IL-10 however, from normal AMs. Curiously, IL-10 and LPS together almost completely inhibited the TNF-R response in sarcoidosis AMs, and this effect was also observed in PBMs at 1 and 10 U/ml IL-10 with LPS (the effect of IL-10 alone in this instance was not determined). A previous study (Joyce *et al.* 1995) has shown that IL-10 can induce the type II TNF-R release in human PBMs, but we were unable to detect any type I TNF-R from normal PBMs. It is suggested by this data that the presence of IL-10 in the sarcoidosis lung could lead to production of TNF-R, thus enhancing the ability of IL-10 to inhibit TNF- α activity in this disease.

These data underline the complex inter-relationship that exists between cytokines in inflammation. We did find elevated TNF- α in the plasma and BALF of sarcoidosis patients in this study, but is not apparently produced by the AMs. TNF-R production is also enhanced, and the AMs may be an important source. In contrast, IL-10 production is not elevated in the BALF, but there is enhanced spontaneous IL-10 production by sarcoidosis AMs and enhanced sensitivity to IL-10 in terms of inhibition of AM TNF- α production. Overall these findings would suggest that TNF- α regulation by IL-10 and TNF-R may be altered, but is not necessarily defective in the lungs of the sarcoidosis patients in this study, but further evaluation with other mediators in the TNF- α network such as IL-13, IL-4 and the type II receptor would give a more complete picture. Study of stage one patients with only a mild degree of disease severity is problematic and thus a broader study involving patients with more advanced disease may be of value to determine whether inappropriate regulation of TNF- α is implicated mainly in the pathogenesis of more aggressive forms of sarcoidosis.

5. TNF- α REGULATION IN CRYPTOGENIC FIBROSING ALVEOLITIS

5.1 Introduction

Cryptogenic fibrosing alveolitis (CFA) is characterised by progressive loss of pulmonary function and fibrosis of the interstitium and it is the most common form of interstitial lung disease, with an annual mortality rate in England and Wales of approximately 1500 (du Bois, 1993). Originally described in 1907 by Sandoz, it has a similar histological appearance to other forms of fibrotic lung disease such as asbestosis, where the aetiology of the disorder is known (Crystal *et al.* 1981a). CFA, however, is triggered by an unknown event leading to the development of a neutrophil alveolitis which precedes the interstitial fibrosis (Crystal *et al.* 1981a; Turner-Warwick *et al.* 1980).

There has been a great deal of speculation as to whether CFA is caused by an immune response to a particular pathogen, such as Epstein Barr Virus (Egan *et al.* 1995), or it is the manifestation of an uncontrolled inflammatory response to a common environmental agent (Libby *et al.* 1983). CFA has been proposed to have an autoimmune component, with as many as 37% of patients testing positive for the presence of autoantibodies, among them rheumatoid factor (Stack *et al.* 1972). Indeed many patients with rheumatoid arthritis develop pulmonary fibrosis which is similar in nature to CFA (Sewell and Trentham, 1993). The presence of circulating immune complexes in CFA patients is also suggestive of autoimmune involvement, and internalisation of these complexes by AMs has been proposed as a mechanism for AM activation seen in CFA (Libby *et al.* 1983). Genetic factors, which support the case for an intrinsic disorder may also be important, since CFA is also known to be associated with the expression of certain HLA markers (Libby *et al.* 1983) and it can also arise as a familial syndrome (Barzo, 1985).

In interstitial lung diseases the cells recovered in the BALF have been found to correlate very closely with the cell populations in the interstitium, where the disease processes are focused (Daniele *et al.* 1985). Indeed, the name interstitial lung disease may in part be a misnomer, since activated effector cells such as AMs, neutrophils and T cells are abundant in the alveolar spaces (Watters *et al.* 1987; Crystal *et al.* 1981a) as well as the interstitium. AMs from CFA subjects are thought to be in an enhanced state of activation, indeed they have been found to produce elevated levels of MIP-1- α (Standiford *et al.* 1993), IL-1- β (Kline *et al.* 1993), TNF- α (Zhang *et al.* 1993) and IL-8 (Carré *et al.* 1991; Libby *et al.* 1983). TNF- α has been shown to be increased in the bleomycin-induced rat model of pulmonary fibrosis (Everson and Chandler, 1992). Indeed, lung injury in animal models of pulmonary fibrosis has been prevented by the use of anti-TNF antibodies (Piguet *et al.* 1989), thus demonstrating a requirement for TNF- α in bleomycin-induced fibrosis. Release of TNF- α by AMs in CFA has also been shown to be elevated (Zhang *et al.* 1993). The timecourse for TNF- α release is such that it may feed back on the AM and promote subsequent release of chemokines such as IL-8 and MCP-1. These cytokines may be important in the development of the PMN influx observed in CFA, although the importance of this is debatable since the PMNs found in the BALF may not correlate with disease outcome (Libby *et al.* 1983). Eosinophils and lymphocytes do correlate, however, with elevated numbers in the BALF associated with accelerated progression of disease (Daniele *et al.* 1985; Watters *et al.* 1987). TNF- α can also affect other cells, stimulating proliferation of fibroblast in conjunction with IL-1 (Elias *et al.* 1988), or promoting the release of other pro-inflammatory cytokines from PMNs, as well as proteases, superoxide and histamine (Libby *et al.* 1983; Thelen *et al.* 1993), which are believed to be involved in the loss of alveolar structure associated with CFA. It seems possible therefore that inappropriate regulation of TNF- α could lead to many of the pathological hallmarks of CFA.

The previous chapters introduced the regulatory cytokine IL-10, which has been shown to inhibit TNF- α release in numerous studies (Ralph *et al.* 1992) as well as the circulating soluble TNF receptor (TNF-R) which has been demonstrated to inhibit TNF- α bioactivity. This study is based upon the hypothesis that TNF- α production/activity may be excessive in the lungs of CFA and this may be a consequence of (1) enhanced

capacity of AMs to produce TNF- α , (2) decreased production and or activity of IL-10 and/or (3) decreased production of TNF-R. Release of TNF- α by AMs was measured, as was TNF-R and IL-10 production. The ability of IL-10 to inhibit TNF- α production and induce TNF-R production by AMs was also investigated. Experiments were duplicated in PBMs to establish whether TNF- α regulation was altered in mature macrophages when compared to their circulating counterparts.

5.2 Experimental Design

AMs and PBMs were isolated from patients with CFA as described previously (Chapter 2). Patients in this study were undergoing fiberoptic bronchoscopy for diagnostic purposes and were not receiving steroid treatment prior to the bronchoscopy. All the subjects had significant pulmonary impairment and transbronchial biopsy revealed chronic inflammatory changes consistent with pulmonary fibrosis.

Purified AMs and PBMs were cultured at a density of 1×10^6 cells per ml in culture medium. Control wells contained medium alone. To eliminate the possibility of endotoxin contamination affecting results, all cell cultures had additional control wells containing 5 µg/ml polymyxin B sulphate (Sigma) which binds to LPS. Cultures which showed a reduced TNF- α response in the presence of polymyxin were considered contaminated and therefore not considered for the study.

LPS was used as an immune response stimulus in our cultures. Dose response curves were utilised to ascertain sensitivity to LPS, but 10 µg/ml LPS was shown to be the optimum concentration and was used as the preferred dose when only one concentration of LPS was appropriate. 10 µg/ml LPS was also selected for PBM cultures, despite an optimum dose of 100 ng/ml LPS, so that both cell species had identical culture conditions so permitting direct comparison. IL-10 was used at a dose range 0.001 - 100 U/ml in isolation and also in conjunction with 10 µg/ml LPS. Supernatants from the cultures were removed after 24 hours and frozen at -70°C until required. Plasma and cell-free BALF were removed from whole blood and total lavage fluid respectively by centrifugation and stored likewise until required. Lavage protein levels were determined as grammes of protein per million recovered BAL cells. Repeated freeze-thaw cycles were avoided to reduce loss of protein activity

5.3 Results

5.3.1 TNF- α in the plasma and BALF of CFA subjects

In normal subjects TNF- α was below the limit of detection for the assay. In contrast, the plasma and BALF of CFA subjects contained considerable amounts of TNF- α , 0.21 ± 0.064 ng/ml and 0.352 ± 0.063 ng/million BAL cells respectively ($p < 0.01$) (Figure 5.1).

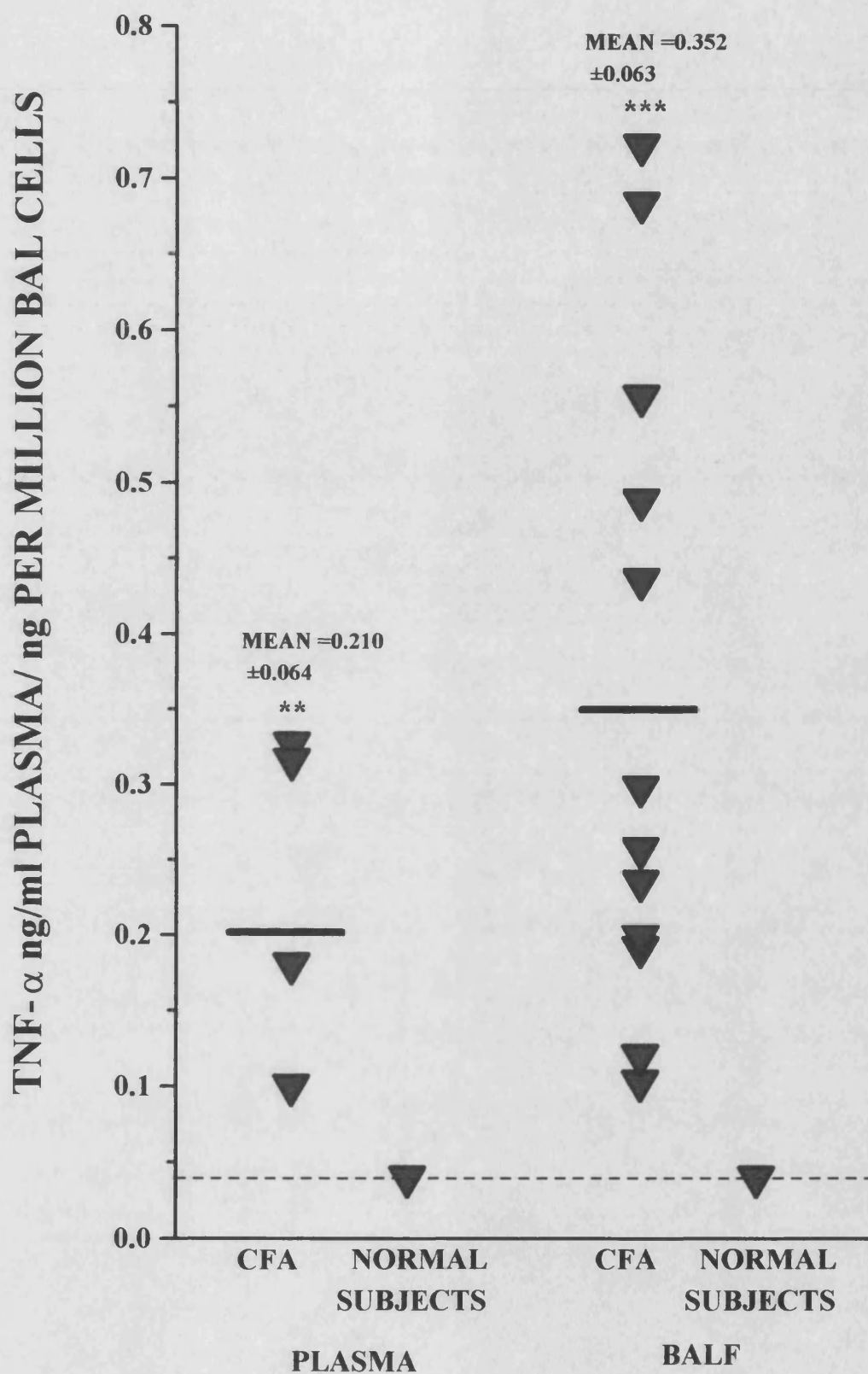
5.3.2 Spontaneous and LPS-induced TNF- α release by AMs and PBMs from CFA subjects

Spontaneous TNF- α release by AMs from normal subjects was comparable to CFA subjects (1.36 ± 0.97 ng/ml and 0.81 ± 0.29 ng/ml respectively), whereas LPS-induced TNF- α in CFA was elevated at all doses of LPS when compared to normal subjects (Figure 5.2a) ($p = 0.053$). Spontaneous release of TNF- α by CFA PBMs was greater than in normal subjects (0.77 ± 0.61 ng/ml and 0.044 ± 0.008 ng/ml respectively), although this difference was not maintained in response to LPS at any dose, with CFA and normal PBMs giving very similar profiles of LPS-induced TNF- α release (Figure 5.2b).

5.3.3 TNF- α inhibition by IL-10 in AMs and PBMs from CFA subjects

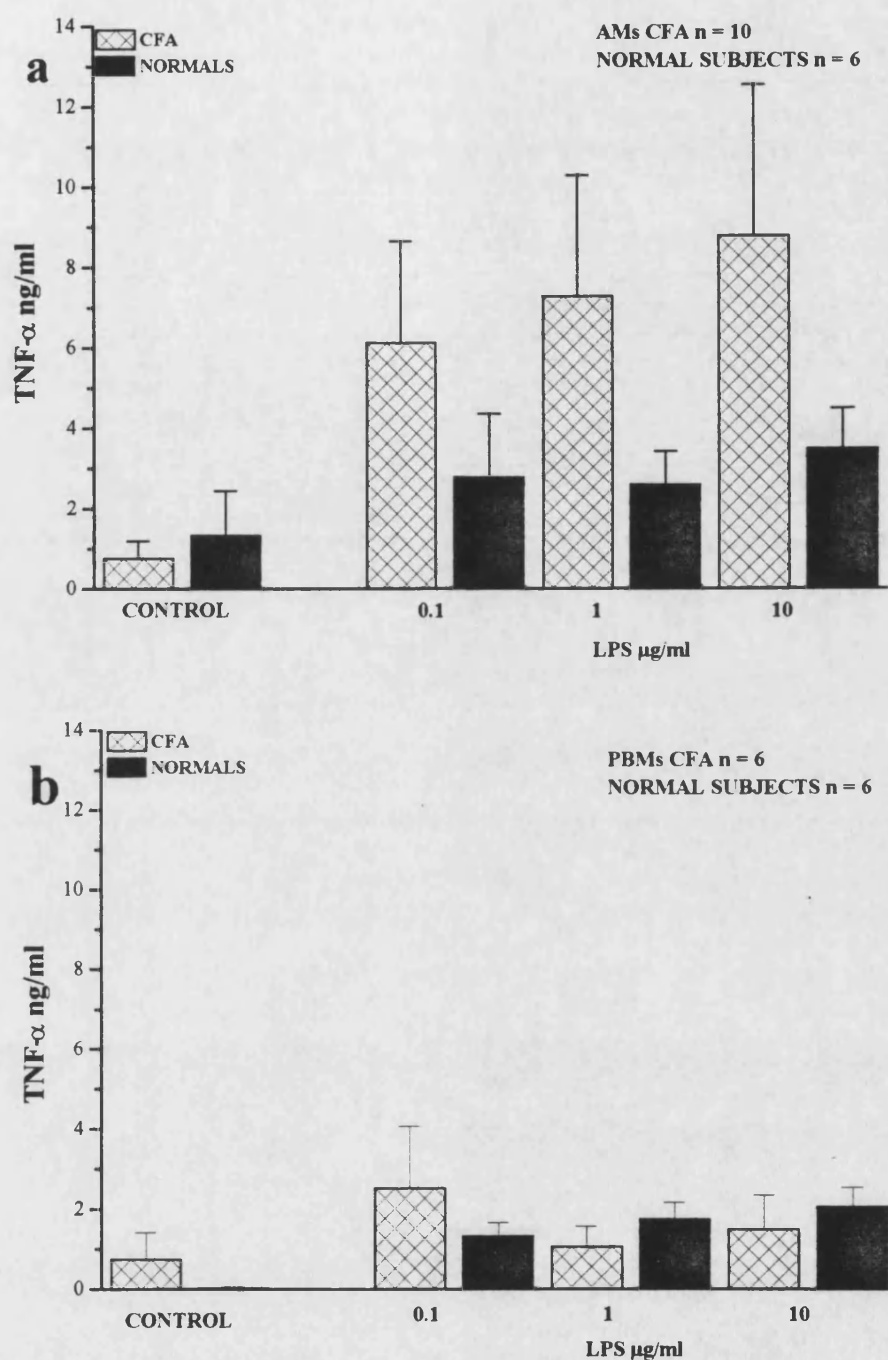
In normal subjects IL-10 reduced LPS-induced TNF- α significantly at doses greater than 10 U/ml. CFA AMs showed a greater sensitivity to IL-10; TNF- α was reduced by IL-10 at all doses, significantly at the lowest dose of 0.001 U/ml IL-10 ($p < 0.05$) (Figure 5.3a). This translated to a percentage reduction of 75.7 ± 20.1 in CFA subjects compared to only 35.3 ± 9.4 % in normal subjects with the higher optimum dose of 100 U/ml IL-10. In contrast, PBMs from normal subjects significantly reduced the TNF- α to basal levels at 50 and 100 U/ml IL-10 ($p < 0.01$), but IL-10 had no effect on TNF- α release by CFA PBMs at any dose (Figure 5.3b).

Figure 5.1 TNF- α DETECTED IN THE PLASMA (n = 4) AND BALF (n =12) OF CFA SUBJECTS



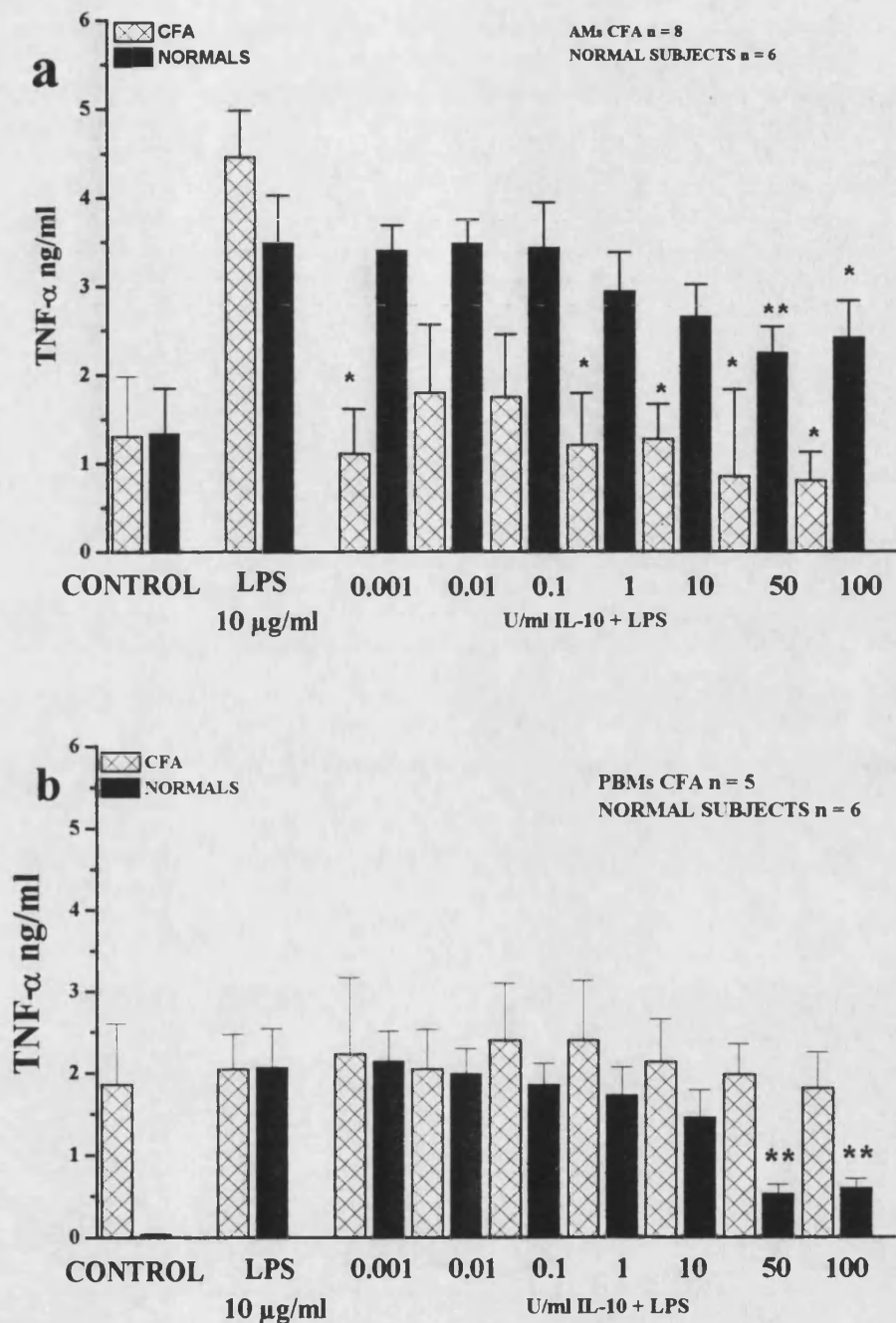
**** $p < 0.01$, *** $p < 0.001$ student's T test**

FIGURE 5.2 THE EFFECT OF LPS ON TNF- α RELEASE BY AMs AND PBMs DERIVED FROM CFA SUBJECTS



AMs (a) and PBM (b) cultured for 24 hours, 1×10^6 per ml, in the presence of a dose range of LPS. Control cells were cultured in medium alone.

FIGURE 5.3 THE EFFECT OF IL-10 ON TNF- α RELEASE BY AMs AND PBMs DERIVED FROM CFA PATIENTS



AMs (a) and PBMs (b) cultured for 24 hours, 1×10^6 cells per ml, with 10 μ g/ml LPS in the presence or absence of a dose range of IL-10. Control cells were cultured in medium alone.

* $p < 0.05$, ** $p < 0.01$ Mann Whitney U test

5.3.4 IL-10 in the plasma and BALF of CFA subjects

IL-10 was detectable in the plasma and BALF of all CFA and normal subjects (Figure 5.4). The plasma values were 32.0 ± 12.88 pg/ml and 65.47 ± 28.26 pg/ml respectively, and the BALF values 389.65 ± 102.15 pg/ml and 334.55 ± 198.16 pg/million BAL cells respectively. There were no statistically significant differences between the groups.

5.3.5 Spontaneous and LPS-induced IL-10 production by AMs and PBMs from CFA subjects

AMs from normal subjects produced a spontaneous level of 12.6 ± 8.4 pg/ml IL-10 which was higher than the 4.02 ± 1.90 pg/ml released spontaneously by CFA AMs (Figure 5.5a). However, LPS-induced IL-10 was elevated in CFA AMs at all doses when compared to normal subjects. Also, PBMs from CFA subjects produced more IL-10 in response to 1 and 10 μ g/ml LPS compared to normal PBMs (Figure 5.5b), and spontaneous IL-10 was once again elevated in CFA subjects, with 26.84 ± 13.1 pg/ml for CFA PBMs compared to 8.71 ± 4.92 pg/ml for normal PBMs.

5.3.6 TNF-R in the plasma and BALF of CFA subjects

TNF-R was detectable in both the plasma and BALF of CFA and normal subjects (Figure 5.6). Plasma TNF-R in CFA subjects was significantly elevated, with 1.621 ± 0.125 ng/ml compared to 0.421 ± 0.055 ng/ml for normal subjects ($p < 0.01$). CFA BALF also contained elevated TNF-R with 1.024 ± 0.396 ng/million BAL cells compared to only 0.254 ± 0.063 ng/million BAL cells for normal subjects, although this failed to reach statistical significance ($p = 0.12$).

5.3.7 Spontaneous and LPS-induced TNF-R release by AMs and PBMs from CFA subjects

In normal subjects spontaneous TNF-R was measured at 0.039 ± 0.017 ng/ml which then declined in response to LPS in a dose dependent manner (Figure 5.7a).

FIGURE 5.4 IL-10 DETECTED IN THE PLASMA (n = 16) AND BALF (n =20) OF CFA SUBJECTS

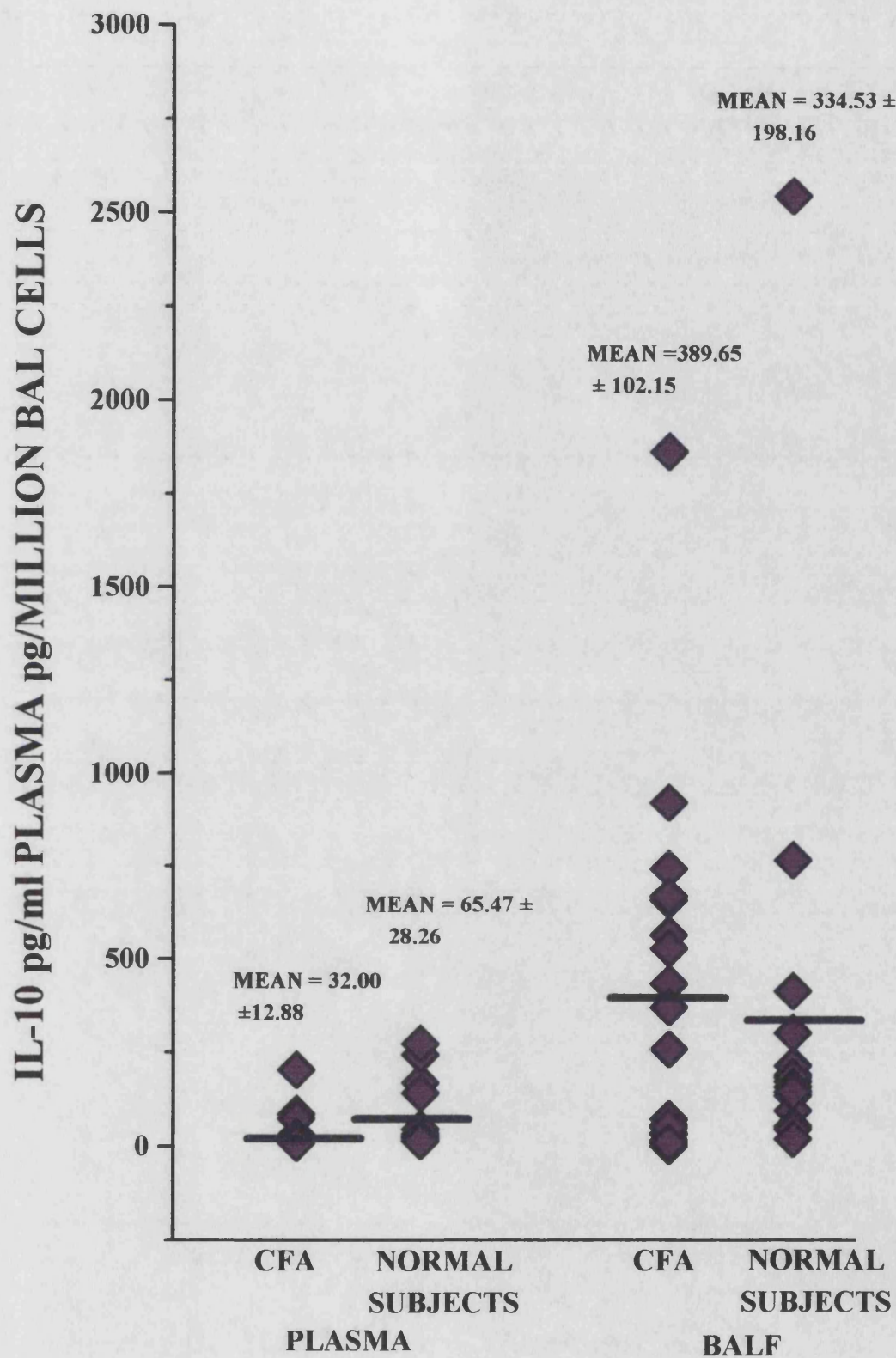
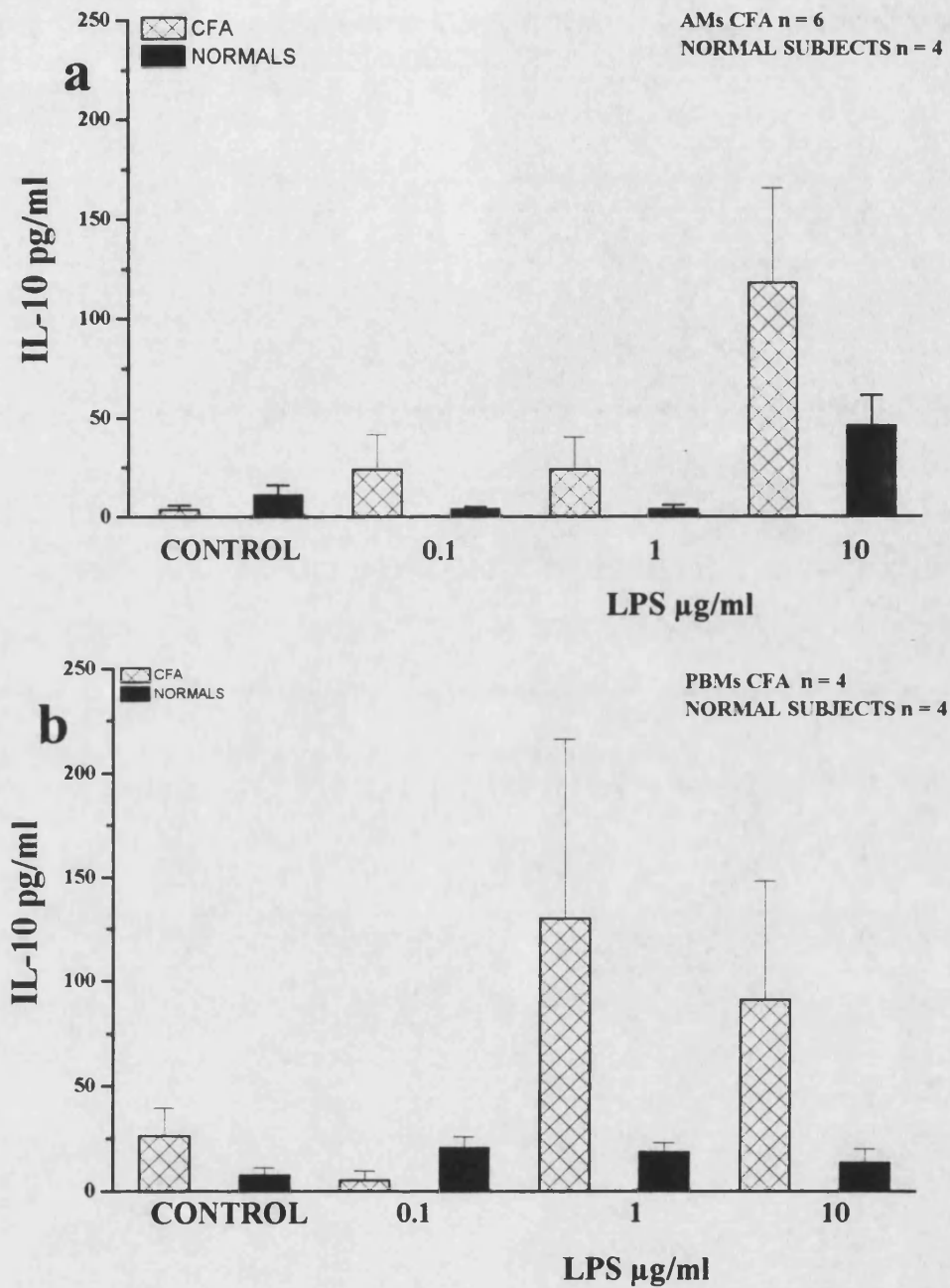
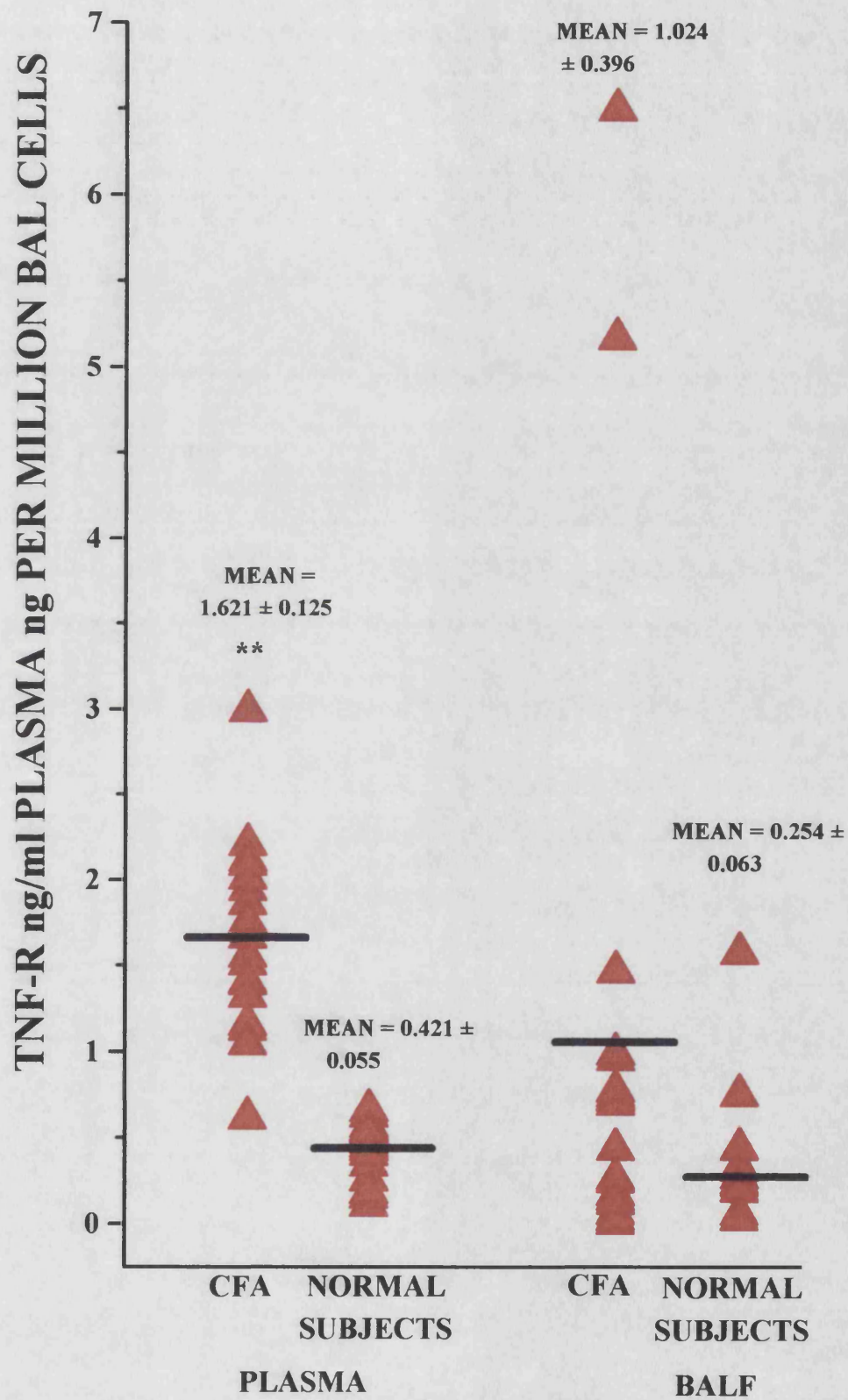


FIGURE 5.5 THE EFFECT OF LPS ON IL-10 PRODUCTION BY AMs AND PBMs DERIVED FROM SUBJECTS WITH CFA



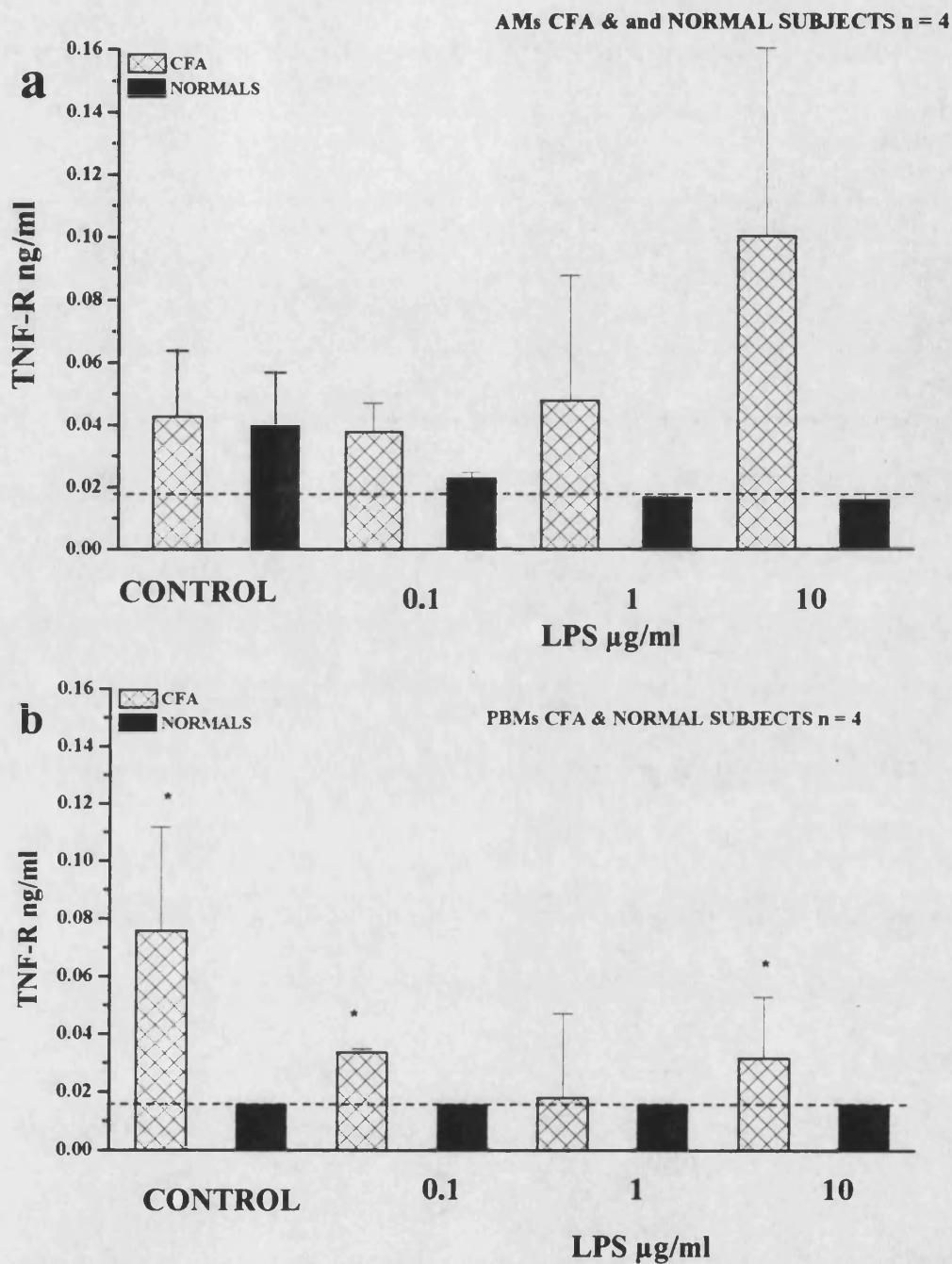
AMs (a) and PBMs (b) were cultured for 24 hours, 1×10^6 cells per ml, in the presence of a dose range of LPS. Control cells were cultured with medium alone.

FIGURE 5.6 TNF-R DETECTED IN THE PLASMA (n=18) AND BALF (n=20) OF CFA SUBJECTS



**** $p < 0.01$ Mann Whitney U test**

Figure 5.7 THE EFFECT OF LPS ON TNF-R PRODUCTION BY AMs AND PBMs DERIVED FROM CFA SUBJECTS



AMs (a) and PBMs (b) cultured for 24 hours, 1×10^6 cells per ml, with a dose range of LPS. Control cells were cultured in medium alone.

- - - detection limit 0.016 ng/ml TNF-R

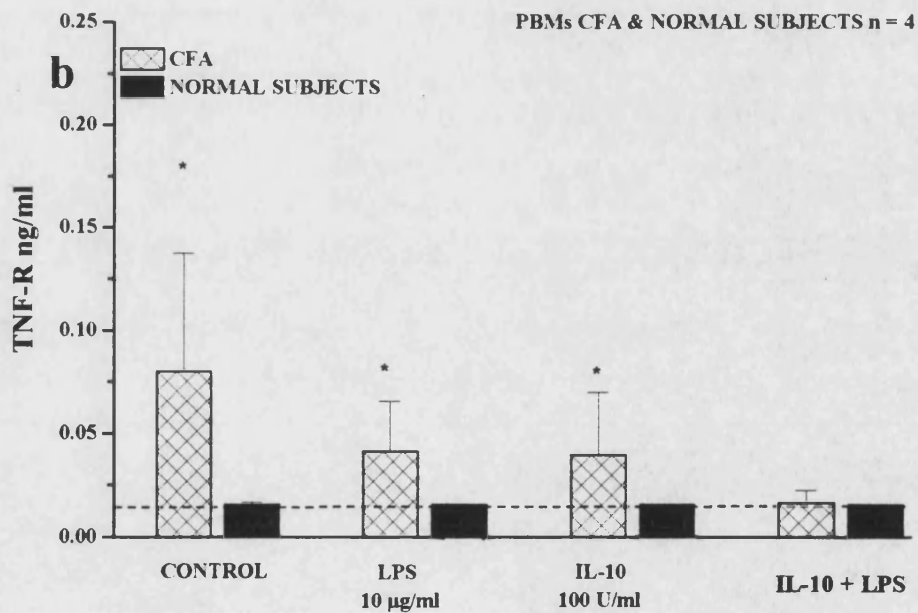
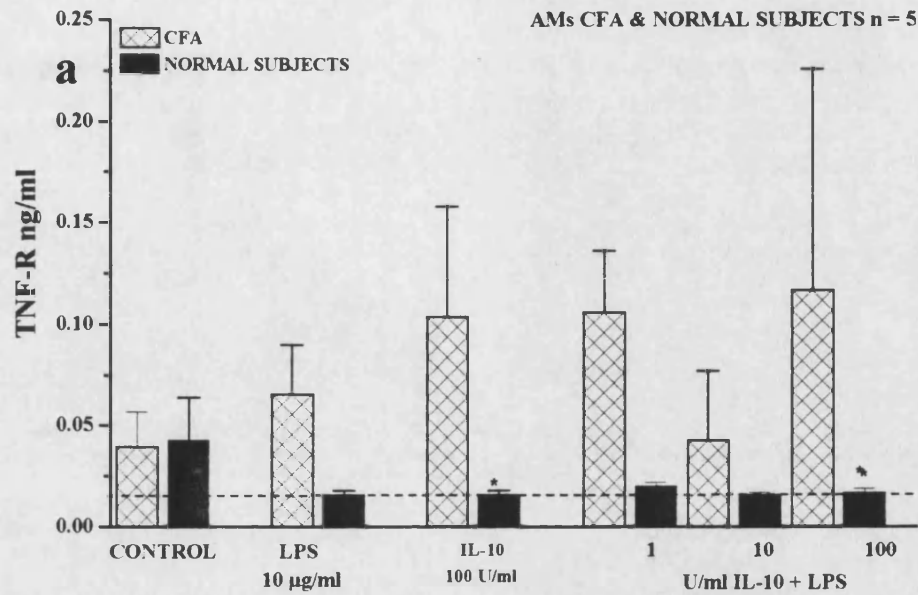
* $p < 0.05$ Mann Whitney U test

Although spontaneous TNF-R from CFA AMs was comparable to normal subjects at 0.042 ± 0.014 ng/ml, no inhibition was demonstrated in response to LPS at any dose. Normal PBMs failed to produce detectable TNF-R at any dose, whereas by contrast PBMs from CFA subjects produced spontaneous TNF-R at 0.751 ± 0.042 ng/ml ($p < 0.05$) which was reduced in response to LPS at doses of 1 and 10 μ g/ml (Figure 5.7b).

5.3.8 The effect of IL-10 on TNF-R release by AMs and PBMs from CFA subjects.

Spontaneous TNF-R levels were significantly reduced by IL-10 alone at 100 U/ml IL-10 in normal AMs ($p < 0.05$), whereas IL-10 was unable to reduce further the reduction in response to 10 μ g/ml LPS (Figure 5.8a). In marked contrast, IL-10 alone increased TNF-R release by CFA AMs from a basal level of 0.047 ± 0.014 ng/ml to 0.104 ± 0.141 ng/ml. IL-10 in combination with LPS did not lead to any further increases. This is in contrast to PBMs from CFA subjects, where LPS and IL-10 reduced TNF-R release when added separately from 0.063 ± 0.047 ng/ml to 0.046 ± 0.027 ng/ml and 0.044 ± 0.031 ng/ml respectively, and when added together they reduced the amount of TNF-R released by CFA PBMs to 0.021 ± 0.009 ng/ml (Figure 5.8b). PBMs from normal subjects failed to produce any detectable TNF-R.

FIGURE 5.8 THE EFFECT OF IL-10 AND LPS ON TNF-R RELEASE BY AMs DERIVED FROM CFA SUBJECTS



AMs (a) and PBMs (b) cultured for 24 hours, 1×10^6 cells per ml plus or minus $10 \mu\text{g/ml}$ LPS and/or 100 U/ml IL-10. Control cells were cultured with medium alone.

--- detection limit 0.016 ng/ml TNF-R

* $p < 0.05$ Mann Whitney U test

5.4 Discussion

These results demonstrate that TNF- α and TNF-R release by AMs and IL-10 and TNF-R release by PBMs derived from CFA subjects are distinct from the cells derived from normal subjects. This is indicative of a disease which is not fully compartmentalised, reflecting inflammatory changes in the blood compartment as well as the lung. As previously shown (Zhang *et al.* 1993), TNF- α levels in CFA were significantly elevated in plasma and BALF when compared to normal subjects. The levels were somewhat higher in the BALF which suggests that much of the TNF- α in the plasma may be pulmonary-derived TNF- α . This elevated plasma TNF- α may explain the increased TNF-R and IL-10 release by PBMs, since TNF- α has been shown to induce production of both these regulatory proteins (Porteau and Hieblot, 1994; Wanidworanun and Strober, 1993).

Spontaneous release of TNF- α was no different from that released by normal AMs, which is contrary to the findings of Zhang *et al.* (1993) who found significant elevations in spontaneous release. This is the only published report examining TNF- α release by AMs in CFA, and different AM purification protocols could possibly lead to varying amounts of *in vitro* cytokine induction, and the elimination of possible endotoxin contamination is crucial. This thesis used polymyxin B in the cultures to detect the presence of endotoxin, and results were only considered valid if cultures were found to be endotoxin-free. There were no such controls published in Zhang's study. Since the data suggests that CFA AMs have a more potent response to LPS in culture, even trace contamination may lead to artificially high control values. In terms of LPS-induced TNF- α release these results do confirm the previous report which demonstrated that TNF- α is elevated compared to normal AMs in response to LPS, although this failed to reach statistical significance due to high variability in the recorded values. It is also a possibility that the TNF- α measured in the BALF was not derived from AMs, since TNF- α can be released from other cellular sources such as PMNs (Xing *et al.* 1993), endothelial cells and T cells (Fiers, 1991). The differential cell counts of the BALF of CFA patients consistently indicated more than thirty per cent PMNs, suggesting that they may be a major source of pulmonary TNF- α in CFA.

TNF- α has been demonstrated to induce many of the pathological hallmarks of CFA, including fibroblast proliferation (Elias *et al.* 1988) and production of collagen type I and II and fibronectin by fibroblasts (Campa 1993), all of which are implicated in the development of tissue fibrosis. TNF- α has also been shown to trigger release of IL-8 and MIP-1- α (Matsushima and Oppenheim, 1989; Wolpe and Cerami, 1989), possibly in an autocrine fashion from AMs, and these chemokines may have a central role in mediating the neutrophil alveolitis characteristic of CFA. IL-8 and MIP-1- α can stimulate neutrophil degranulation releasing collagenase, elastase and superoxide anions, which are able to contribute to tissue injury (Wolpe and Cerami, 1989; Cohen *et al.* 1988). In this scenario it is possible that elevated TNF- α may be a main contributor to the alveolitis and tissue fibrosis seen in CFA.

In normal subjects IL-10 was released spontaneously by AMs in small amounts, and this was increased in response to LPS at 10 μ g/ml. In comparison AMs from CFA subjects produced more IL-10 than normal subjects in response to LPS, although spontaneous levels were comparable. IL-10 was also not apparent in elevated amounts in the plasma or BALF of CFA subjects. Raised IL-10 has been reported to be a control measure to counter-act the high levels of TNF- α in inflammatory disease (Katsikis *et al.* 1994), but this does not appear to be the case with CFA, although it needs to be emphasised that IL-10 was determined at the optimum time point for TNF- α detection rather than the optimum for IL-10 itself so this may not be an accurate interpretation. It has been demonstrated that TNF- α release from PBMs has been able to stimulate IL-10 in an autocrine fashion (Wanidworanun and Strober, 1993) and this may be an important mechanism for TNF- α regulation within the cytokine network. IL-10 has also been shown to be elevated in another chronic inflammatory disease, rheumatoid arthritis (Katsikis *et al.* 1994), and inhibition of IL-10 by anti-IL-10 antibody leads to increased levels of TNF- α in the synovial fluid, which is demonstrative of a feedback mechanism controlling TNF- α production involving IL-10. This data suggests that although production of IL-10 does not appear to be compromised in CFA, there is no elevated production of IL-10 in response to the TNF- α . Interestingly, IL-10 does demonstrate a greater potency on TNF- α inhibition in these cells. In normal AMs IL-10 at the optimum dose of 100 U/ml gave a percentage reduction of approximately 35%. In contrast AMs

from CFA subjects gave more than an 70% reduction in TNF- α in response to an IL-10 dose as low as 0.001 U/ml. There is little known about the IL-10 receptor on human cells at present, but the increased efficacy of IL-10 on AMs in CFA could be explained by an increased density of IL-10 receptor on these cells. The apparently increased potency may also be explained by autocrine feedback of intrinsic IL-10 on the AM and is supported by the increased production of IL-10 by CFA AMs in response to LPS. The use of anti-IL-10 antibody in the cultures would have addressed this question since it would have inactivated the biological effects of any intrinsically produced IL-10, so identifying any additive effects it may have in association with extrinsic IL-10.

This leads to the role of TNF-R in CFA, which has been previously been demonstrated to inhibit TNF- α activity. TNF-R was found to be elevated in the plasma and BALF of CFA subjects, significantly so in the former. This finding is accentuated by the greatly increased TNF-R released by CFA PBMs, compared to normal PBMs which released no measurable TNF-R, either spontaneously or in response to LPS. This may be a response to the elevated TNF- α which may be leaking into the plasma of CFA subjects. Spontaneous release from CFA AMs was comparable to normal subjects, but the response to LPS was quite distinct. In normal AMs TNF-R was decreased in a dose-dependent manner in response to LPS. This effect was not observed in CFA subjects. This could possibly explain the enhanced capacity of AMs from CFA subjects to release TNF-R, but it is important to consider that the time point for TNF-R determination may not have been appropriate and that CFA AMs do not produce more TNF-R, but have a more rapid response to stimulus. It is not presently known whether maintained production of TNF-R in CFA is a consequence of enhanced TNF- α release, but it is possible that chronic release of TNF- α may trigger receptor shedding in order to dampen down the inflammatory response. Indeed it has been demonstrated with IL-1 and IL-1ra (Galve-de Rochemonteix *et al.* 1993), as well as TNF- α and TNF-R (Porteau and Hieblot, 1994) that receptor shedding can be stimulated by the corresponding cytokine, and elevated TNF-R has been detected in the plasma of patients with endotoxaemia, who have very high circulating TNF- α levels (Spinass *et al.* 1992). In contrast, it seems that normal AMs which are not chronically activated reduce their production of soluble receptor, perhaps permitting a "no holds barred" inflammatory response. IL-10 is also

implicated in TNF-R shedding; 100 U/ml IL-10 triggered release of TNF-R in CFA AMs, whereas it actually inhibited TNF-R release by normal AMs. This effect was not seen in PBMs, where LPS and IL-10, both together and separately inhibited TNF-R release. A previous study on normal human PBMs found that they did shed increased amount of the type II TNF-R, but they support this data suggesting that the type I receptor shedding could not be measured (Joyce *et al.* 1995), since it is present at a much lower density.

Overall these results indicate that AMs from CFA subjects may be in an enhanced state of activation. It appears that AMs from CFA subjects do not have a reduced capacity to release IL-10 and TNF-R, and indeed they may have an increased capacity to produce the latter. It seems that these mechanisms themselves may not be sufficient to control the TNF- α response fully, and that it is likely that other unexplored mechanisms are involved, possibly IL-4 and IL-13. An alternative interpretation would be that the elevated TNF-R could have pathological consequences. In inflammatory disease the initial response may be defective, possibly due to inappropriate production or activity of cytokine inhibitors which prevent an acute “no holds barred” response and thus leads to a chronic state of activation, whereas in normal subjects the initial inflammatory response may be more potent, allowing for a more rapid resolution.

6. TNF- α REGULATION IN ADULT RESPIRATORY DISTRESS SYNDROME

6.1 Introduction

The Adult Respiratory Distress Syndrome (ARDS) was first recognised in 1967, when Ashbaugh and co-workers observed acute respiratory failure in twelve patients on the intensive care unit. They reported a mortality rate of 58%, which in the subsequent quarter of a century has only moderately improved, indeed a recent study in the UK estimating mortality from ARDS to be 38% (Webster *et al.* 1988). It has been suggested that improvements in life support technology are not sufficient to improve the prognosis for patients with ARDS, and that only a more thorough understanding of the pathophysiology of this acute pulmonary disease will have any major impact on patient survival.

ARDS may be regarded as the final common pathway for many causes of acute lung injury, with its very rapid progression to obliterative pulmonary fibrosis, confirmed by post-mortem examination within three weeks of ARDS onset (Pratt *et al.* 1979). ARDS has often been compared to CFA since both diseases are characterised by neutrophil alveolitis, which may precipitate the development of tissue fibrosis (Fowler *et al.* 1987; Miller *et al.* 1992; Repine, 1992). However, ARDS and CFA are distinct since the former is very rapidly debilitating, whereas CFA may take many years to progress to life-threatening loss of pulmonary function. In addition, it has been observed that apparent fibrosis in ARDS may be reversible in more than 60% of survivors (Elliott *et al.* 1987; Rinaldo and Rogers, 1982) and this feature of the disease is of great interest since it is one of the few examples of a resolution of fibrotic injury, and although fibrosis can be arrested in CFA it has never been shown to completely resolve. This could be due to differences in the nature of the fibrotic process i.e. the collagen laid down may be altered, but it may also be a reflection of alterations in the inflammatory cascade which precedes the fibrosis.

ARDS is strongly associated with sepsis syndrome (Marks *et al.* 1990; Webster *et al.* 1988) and there is a likelihood that gram-negative endotoxin may trigger the inflammatory events associated with ARDS. Indeed, in animal models of acute lung injury, an infusion of endotoxin evokes an injurious response (Tracey *et al.* 1987a; Rabinovici *et al.* 1994; Eichaker *et al.* 1991). Such models have highlighted a putative role for TNF- α in lung injury since they have demonstrated elevated levels of circulating TNF- α (Eichaker *et al.* 1991; Rabinovici *et al.* 1994). Also, in animal studies of *E. coli* endotoxaemia use of anti-TNF antibodies can prevent lethality (Windsor *et al.* 1993; Tracey *et al.* 1987a). Anti-TNF antibodies have also been administered in clinical trials to sepsis patients with mixed results (Windsor *et al.* 1993). In human studies, endotoxin infusions have also been shown to result in elevated plasma levels of TNF- α appearing as early as sixty minutes after infusion and declining by 4 hours post-infusion (Michie *et al.* 1988). Such studies have highlighted the transient nature of TNF- α in inflammation and could explain much conflicting data regarding the role of TNF- α in acute inflammation and ARDS, although a single bolus of LPS was administered and this is not the scenario in sepsis where LPS can be continually released by gram negative bacteria. Some studies have detected elevated levels of circulating TNF- α in ARDS subject compared to patients with pre-disposing risk factors for ARDS (Marks *et al.* 1990; Chollet-Martin *et al.* 1994; Suter *et al.* 1992), but others have found no difference (Parson *et al.* 1992; Roten *et al.* 1991), and such discrepancies may be due to the timing of samples, since peaks of TNF- α production may mirror the peaks and troughs of LPS plasma levels in the sepsis patient. Since ARDS is primarily a pulmonary disease, it is probably more relevant to measure TNF- α in the BALF, and Millar and co-workers (1989) did find elevated TNF- α in the bronchopulmonary secretions of 5 patients with ARDS compared to at risk patients. The probable source of this TNF- α production in ARDS has been identified as the AM (Tran Van Nhieu *et al.* 1993; Suter *et al.* 1992).

It is not surprising that TNF- α should be implicated in the pathophysiology of ARDS since many of the features of the disease can be mediated by this cytokine. Vascular endothelial permeability and endothelial cell damage are features of ARDS (Braude *et al.* 1986; Bone, 1993), contributing to the non-cardiogenic pulmonary oedema characteristic of the disease. TNF- α has been shown to enhance vascular endothelial barrier

permeability *in vitro* (Nawroth and Stern, 1986). Another cytokine, IL-8 is elevated in ARDS (Cohen *et al.* 1988; Miller *et al.* 1992), and Donnelley and co-workers (1993) have reported that AM-derived IL-8 may be a marker of ARDS progression in at risk subjects. TNF- α is known to stimulate IL-8 release from AMs and fibroblasts (Rolfe *et al.* 1991) and thus may contribute indirectly to the neutrophil infiltration and degranulation mediated by IL-8 in ARDS. This may be an important mechanism in ARDS since neutrophil-derived hydrogen peroxide, oxygen radicals and elastase, all of which are elevated in the BALF of ARDS subjects are believed to have an important role in the obliterative alveolitis and fibrosis seen in the ARDS lung (Carré *et al.* 1991; Repine, 1992; Rocker *et al.* 1989; Lee *et al.* 1981; Hällgren *et al.* 1987). Pulmonary fibrosis in ARDS is indicated by elevated levels of AM-derived basic fibroblast growth factor (bFGF) (Henke *et al.* 1993) and increased collagen deposition (Rinaldo and Rogers, 1982). TNF- α is known to promote the laying down of collagen fibres by fibroblasts (Campa 1993) as well as promoting fibroblast proliferation *in vitro* (Elias *et al.* 1988).

The known pathophysiological effects of TNF- α described above would suggest that inappropriate regulation of this mediator may lead to lung injury, and this raises the hypothesis that TNF- α regulation in ARDS may be inadequate. This could be due to an alteration in the relationship of TNF- α to regulatory proteins such as soluble receptors, receptor antagonists or cytokines. IL-10 is one such cytokine which has demonstrated an ability to inhibit TNF- α production. The circulating soluble receptor, although not known to inhibit production can inhibit biological activity. This study has attempted to investigate the source of the TNF- α and how it may be regulated. ARDS subjects were compared to subjects with pre-disposing risk factors, to determine any differences in TNF- α activity which may be responsible for ARDS progression. AMs and PBMs were cultured, and the supernatants analysed for TNF- α protein. TNF- α was also measured in the BALF and plasma. IL-10 production and its impact on TNF- α production by AMs and PBMs was also measured. Finally TNF-R in the BALF and plasma as well as production by AMs and PBMs, was measured to establish any inhibition of TNF- α protein by the soluble receptor.

6.2 Experimental Design

Thirty four patients on the intensive therapy unit (ITU) were included in this study. Patients were eligible for inclusion if they had any of the following pre-disposing risk factors:

Pneumonia

Sepsis Syndrome

Perforated Bowel

Multiple Blood Transfusion

Gastric Aspiration

Multiple Trauma

It would have been desirable to include ventilated controls in this study since normal volunteers could not be directly compared to ARDS subjects as the effects of ventilation on lung physiology could not be taken into consideration. Such controls were unavailable however, so ARDS subjects were only compared to other ventilated patients who had pre-disposing risk factors. Patients were considered to have ARDS if their Murray lung injury score exceeded 2.5. The Murray score (Murray *et al.* 1988) is based on:

- (1) Presence of alveolar infiltrate on x-ray
- (2) Degree of hypoxia
- (3) Reduced pulmonary compliance
- (4) Normal capillary wedge pressure

BAL and venepuncture was performed on all patients. A total of 160 mls of sterile buffered saline was instilled in 20 ml aliquots into the lung and aspirated into chilled siliconised bottles. The BALF was processed as previously described in Chapter 2. Blood was collected from an arterial line into heparinised tubes. Cells were cultured for a period of twenty four hours since the restricted number of AMs returned by BAL on ITU patients meant that only one time point, i.e. the optimum time for TNF- α

production, could be selected. ELISAs were performed for IL-10, TNF- α and TNF-R as previously described in Chapter 2.

6.3 Results

6.3.1 TNF- α in the plasma and BALF of ARDS and at risk subjects

TNF- α was found to be present in most samples tested. In the plasma samples the mean value for TNF- α in ARDS subjects was 0.472 ± 0.237 ng/ml compared to a value of 0.241 ± 0.078 ng/ml for at risk subjects (Figure 6.1). This represented an increase in ARDS plasma TNF- α , which did not reach significance. There was also an elevation in the TNF- α in the BALF of ARDS subjects compared to at risk subjects (0.187 ± 0.132 ng/million BAL cells and 0.026 ± 0.008 ng/million BAL cells respectively).

6.3.2 Spontaneous and LPS-induced TNF- α release from AMs and PBMs

Spontaneous TNF- α release by AMs from ARDS subjects was greater than at risk subjects and this elevation was maintained in response to LPS, where 10 μ g/ml LPS gave 1.53 ± 0.86 ng/ml TNF- α in ARDS subjects compared to only 0.21 ± 0.06 ng/ml for at risk subjects (Figure 6.2a). A similar pattern for TNF- α release was exhibited by PBMs (Figure 6.2b), where 10 μ g/ml LPS stimulated a significantly greater release of TNF- α from PBMs from ARDS subjects compared to those at risk ($p < 0.01$). There was no apparent difference in spontaneous release. Overall TNF- α release by at risk AMs and PBMs was very low.

6.3.3 Inhibition of LPS-induced TNF- α production by IL-10

In ARDS subjects IL-10 reduced LPS-induced TNF- α at all doses, with the exception of 50 U/ml IL-10 (Figure 6.3a). Due to a large degree of sample variation however, there was no significant reduction observed at any dose. LPS-induced TNF- α was very low in the AMs of at risk subjects, with only 0.21 ± 0.06 ng/ml produced. This was not reduced significantly by IL-10 at any dose, although there were apparent reductions at 0.1, 1 and

**Figure 6.1 TNF- α DETECTED IN THE PLASMA AND BALF
OF SUBJECTS WITH ARDS (n = 16) AND AT RISK OF ARDS (n = 17)**

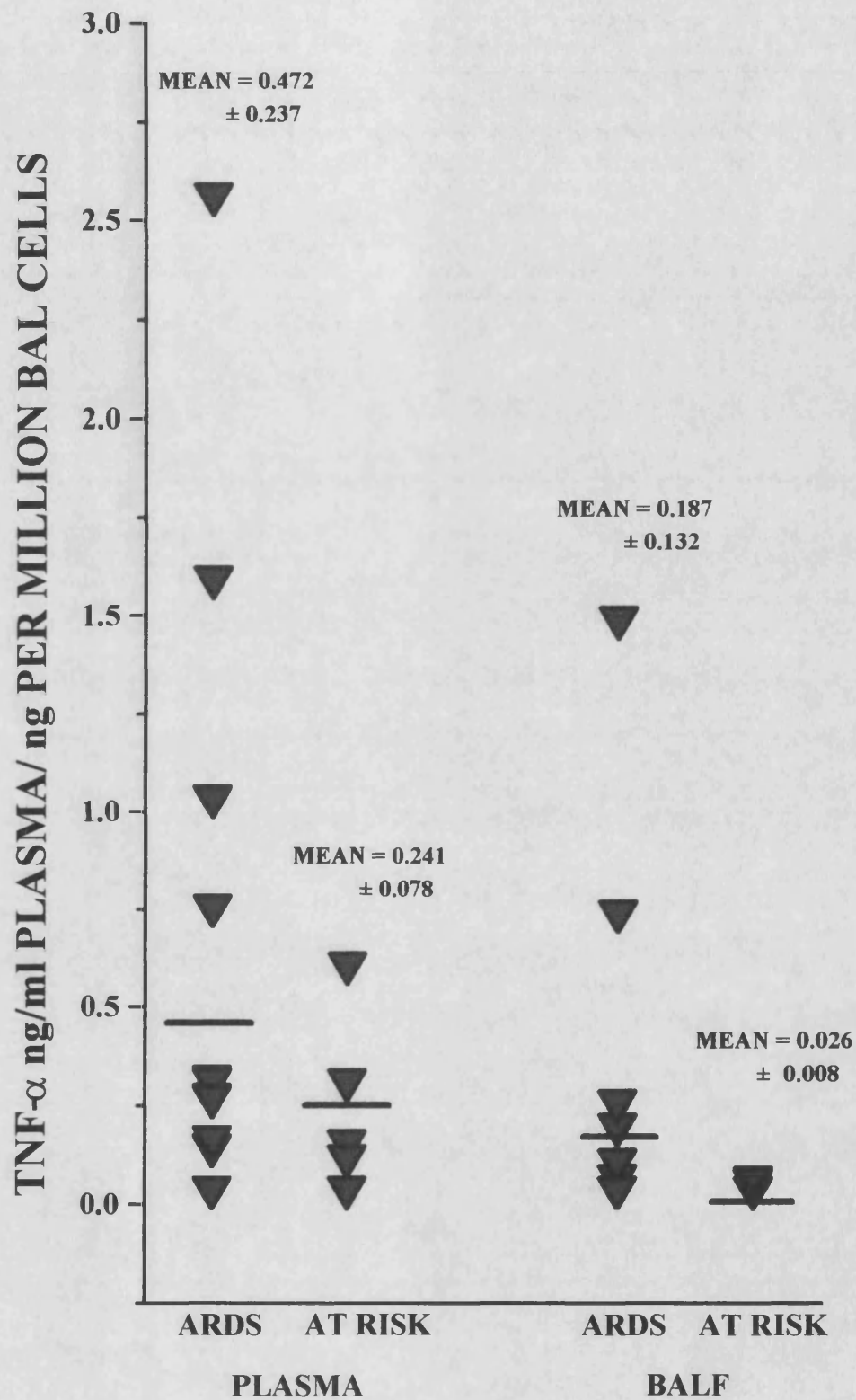
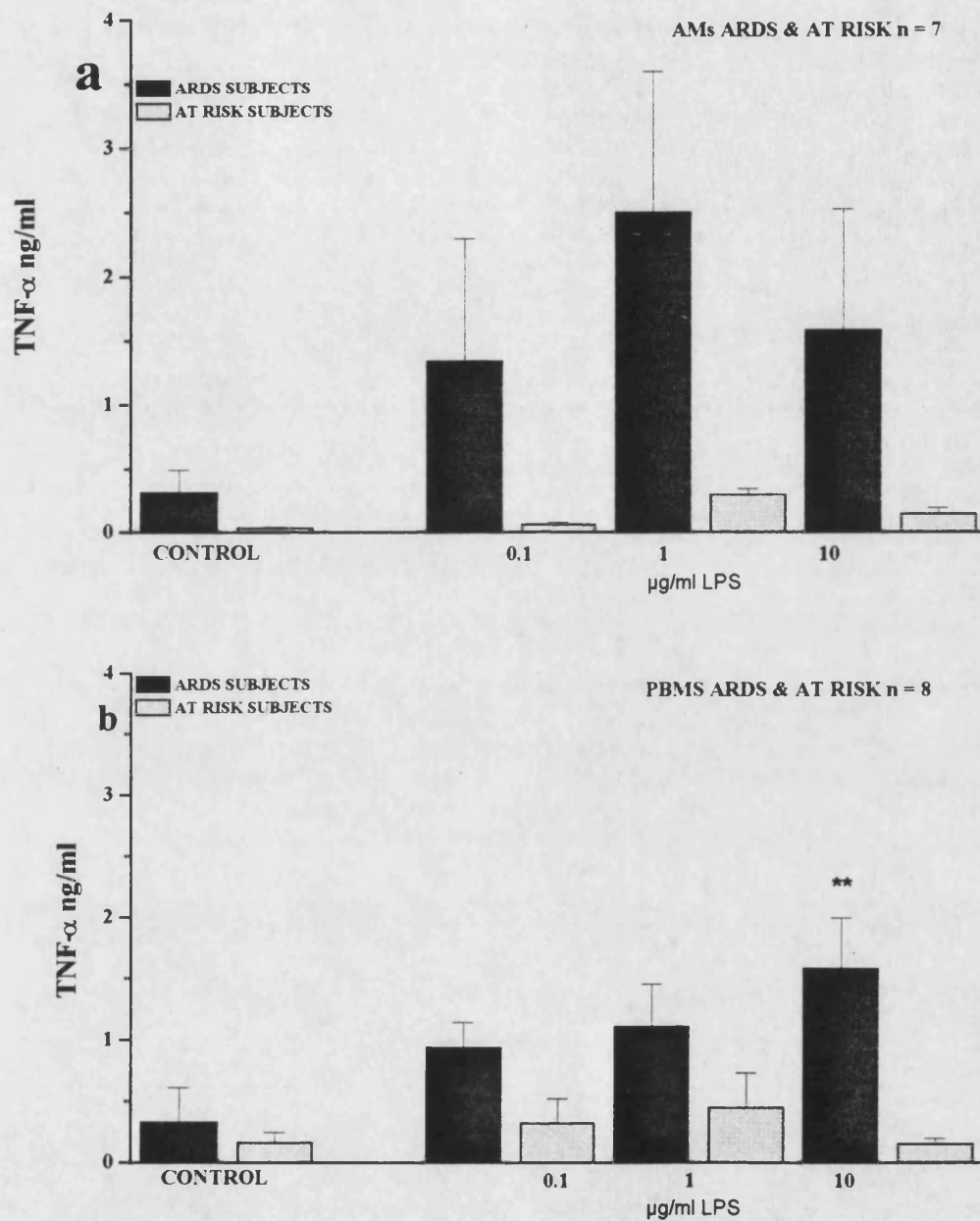


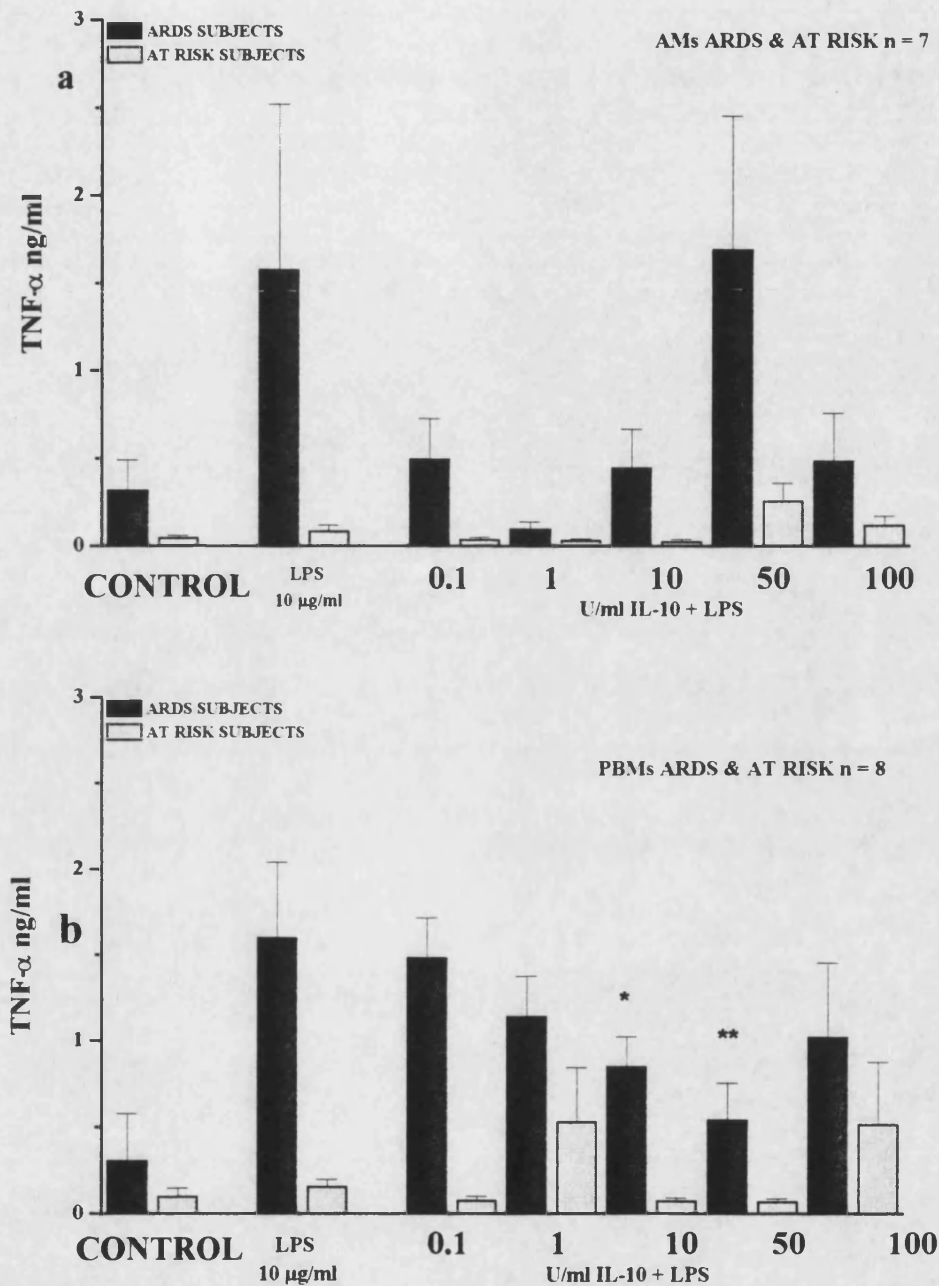
Figure 6.2 SPONTANEOUS AND LPS-INDUCED TNF- α RELEASE FROM AMs AND PBMs DERIVED FROM ARDS AND AT RISK SUBJECTS



AMs (a) and PBMs (b) were cultured for 24 hours, 1×10^6 cells per ml with a dose range of LPS. Control cells were cultured with medium alone.

** $p < 0.01$ Mann Whitney U test

Figure 6.3 THE EFFECT OF IL-10 ADMINISTRATION ON LPS-INDUCED TNF- α RELEASE FROM AMs AND PBMs DERIVED FROM ARDS AND AT RISK SUBJECTS



AMs (a) and PBMs (b) cultured for 24 hours, 1×10^6 cells per ml, with $10 \mu\text{g/ml}$ LPS in the presence or absence of a dose range of IL-10. Control cells were cultured in medium alone.

* $p < 0.05$, ** $p < 0.01$ Mann Whitney U test

10 U/ml IL-10. The top doses of 50 and 100 U/ml had no effect. PBMs from ARDS subjects were much more responsive to IL-10, with a significant reduction occurring in response to 10 and 50 U/ml IL-10, corresponding to an approximate reduction in TNF- α protein of 60% in response to the latter dose ($p < 0.05$) (Figure 6.3b). The top dose of 100 U/ml IL-10 had no statistically significant effect. PBMs from at risk subjects once again produced very little TNF- α in response to LPS, and although there was a suggested reduction in response to 0.1, 10 and 50 U/ml IL-10, these were not significant.

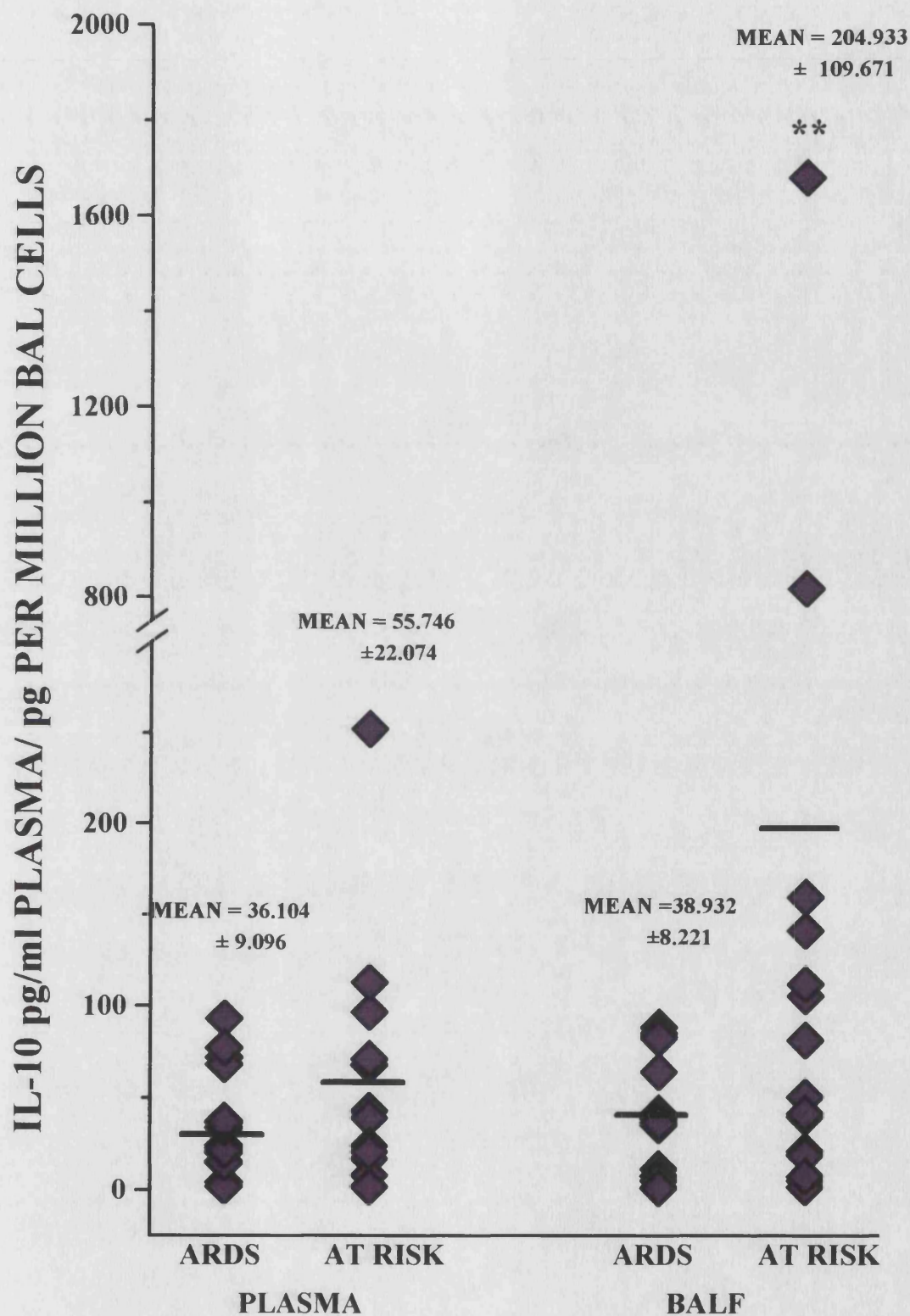
6.3.4. IL-10 in the plasma and BALF of ARDS and at risk subjects

IL-10 was detectable in all plasma and BALF samples of both patient groups (Figure 6.4). Plasma values were 36.104 ± 9.096 pg/ml in ARDS subjects compared to 55.746 ± 22.074 pg/ml for at risk subjects. In the BALF, however IL-10 was diminished in ARDS subjects where there was only 38.93 ± 8.22 pg/million BAL cells IL-10 compared to 204.9 ± 109.67 pg for at risk subjects ($p < 0.01$).

6.3.5 Spontaneous and LPS-induced IL-10 release by AMs and PBMs

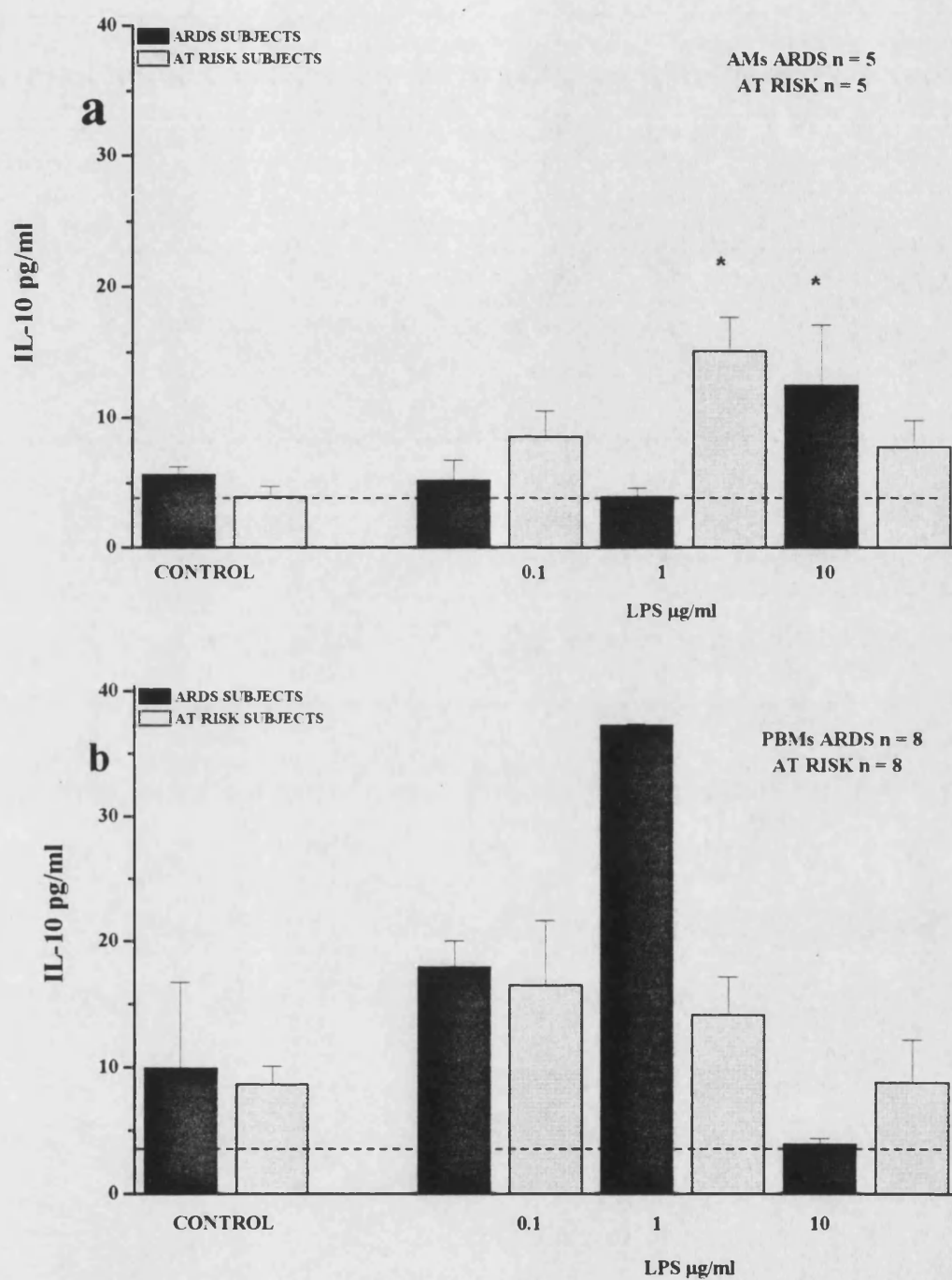
AMs from both patient groups produced IL-10 spontaneously in culture, although less than 5 pg/ml. IL-10 production was significantly enhanced in ARDS AMs in response to 10 μ g/ml LPS ($p < 0.05$), the AMs from at risk subjects responded significantly only to 1 μ g/ml LPS ($p < 0.05$) (Figure 6.5a). PBMs from both patient groups produced more IL-10 than AMs, both spontaneously and in response to LPS. ARDS PBMs exhibited a three-fold increase in IL-10 production in response to 1 μ g/ml LPS, although this was not apparent when the dose was increased to 10 μ g/ml (Figure 6.5b). The optimum dose of LPS for IL-10 production by at risk PBMs was 0.1 μ g/ml, and this declined in response to higher doses.

**Figure 6.4 IL-10 DETECTED IN THE PLASMA AND BALF
OF SUBJECTS WITH ARDS (n = 16) AND AT RISK OF ARDS (n = 17)**



**** $p < 0.01$ Mann Whitney U test**

Figure 6.5 SPONTANEOUS AND LPS-INDUCED IL-10 RELEASE BY AMs AND PBMs DERIVED FROM SUBJECTS WITH ARDS AND AT RISK OF ARDS



AMs (a) and PBMs (b) cultured for 24 hours, 1×10^6 cells per ml, with a dose range of LPS. Control cells cultured in medium alone.

- - - detection limit 4 pg/ml IL-10

* $p < 0.05$ Mann Whitney U test

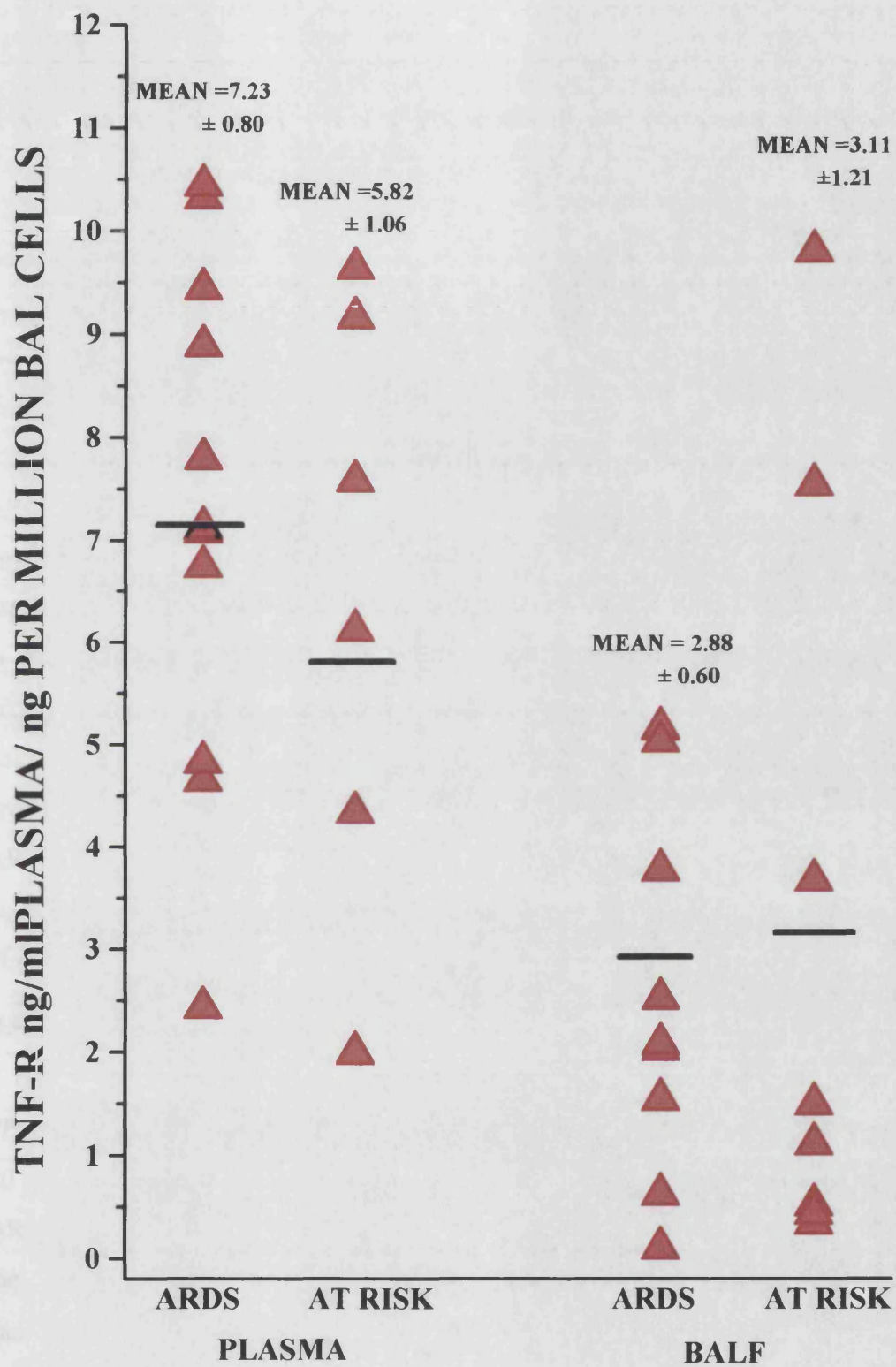
6.3.6 TNF-R in the plasma and BALF of ARDS and at risk subjects

TNF-R was detectable in all plasma and BALF samples of both patient groups. ARDS plasma contain 7.23 ± 0.80 ng/ml TNF-R compared to 5.82 ± 1.06 ng/ml for at risk subjects (Figure 6.6). The BALF values for ARDS and at risk subjects groups were also comparable with 2.88 ± 0.60 ng/million BAL cells and 3.11 ± 1.21 ng/million BAL cells TNF-R respectively.

6.3.7 TNF-R production by AMs and PBMs from ARDS and at risk subjects

TNF-R was below the detection limit in all supernatants from both AMs and PBMs derived from ARDS and at risk subjects (data not shown).

**Figure 6.6 TNF-R DETECTED IN THE PLASMA AND BALF
OF SUBJECTS WITH ARDS (n = 10) AND AT RISK OF ARDS (n = 8)**



6.4 Discussion

The TNF- α profile of AMs derived from patients with ARDS indicates that they may be able to produce more TNF- α than the at risk subjects. This is reflected by the increased levels of TNF- α in the BALF of ARDS subjects and may suggest that regulation of TNF- α in ARDS subjects is abnormal. Care must be taken when interpreting this data however since a number of factors need to be considered. The elevated TNF- α in ARDS subjects may not be a feature of the disease process itself, but a result of direct injury to the lung. It has been demonstrated that the risk of developing ARDS is greater if the injury is direct (Fowler *et al.* 1983) and that cytokine levels are higher in ARDS with pneumonia than without (Jacobs *et al.* 1989). Since all our pneumonia subjects had ARDS it is difficult to conclude whether the TNF- α elevation is due entirely to ARDS or largely as a result of pneumonia. It would therefore be useful in further studies to treat the pneumonia group as a separate entity. The fact that there is slightly elevated TNF- α in the plasma of ARDS patients as well as the BALF may indicate that a systemic process is involved however and it is unlikely to be explained by TNF- α entering the pulmonary circulation from the lung compartment since PBMs from ARDS subjects also produced significantly more TNF- α in response to LPS. Since overall TNF- α release by ARDS AMs (and PBMs to a lesser extent) was greater than the at risk subjects this highlights inappropriate TNF- α regulation as a possible mechanism for the development of ARDS in patients at risk.

IL-10 was investigated as a potential key player in the regulation of TNF- α release, since it has been shown to down-regulate TNF- α production by human macrophages and PBMs (Bogdan and Nathan, 1993; Ralph *et al.* 1992). It was interesting to find that IL-10 was not particularly effective at inhibiting LPS-induced TNF- α release by the AMs of ARDS and at risk subjects. This was not a surprising finding in the at risk subjects, since the window for IL-10 inhibition of TNF- α production was so narrow, but the same cannot be said for the ARDS subjects, where TNF- α levels were higher. This raises the possibility that resistance to IL-10 may explain the increase in TNF- α release by ARDS AMs relative to at risk subjects. It should also be considered however that the timing of the BAL procedure in ARDS patients may be important. TNF- α may peak very early in

the development of ARDS and thus it is conceivable that the initial inhibition of TNF- α by IL-10 is strong, but is then quickly down-regulated in response to over-production of IL-10 or TNF- α . This possibility could be explored by more performing bronchoscopy at regular time intervals, but repetitive bronchoscopy can potentially induce lung injury so less invasive AM retrieval techniques such as tracheal suction would have to be employed.

In order to establish an autocrine role for the effect of IL-10 on TNF- α , IL-10 was measured in AM supernatants. Interestingly, IL-10 production was depressed in the AMs of both patient groups, indicating a possible defect in IL-10 production which may account somewhat for the inflammatory disease seen in ARDS. Although the 24 hour timepoint selected for supernatant collection may not be ideal for IL-10, which peaks at 24-48 hours in PBMs (Wanidworanun and Strober, 1993), this is an unlikely explanation for the findings since when plasma and BALF were examined for IL-10 the ARDS patients had less IL-10 in both fluids than at risk subjects. ARDS BALF contained significantly less IL-10 than normal BALF. A more detailed study of the time course of IL-10 release by AMs from ARDS subjects, as well as studying the changes over time in the disease process by tracheal suction retrieval would be desirable to evaluate further IL-10 production in ARDS. IL-10 is presumed to have a largely anti-inflammatory role, having been shown to inhibit pro-inflammatory cytokine release (Ralph *et al.* 1992) and to down regulate class II MHC and B7 which are important molecules in antigen presentation (de Waal Malefyt *et al.* 1991b; Ding *et al.* 1993b). IL-10 has been found to be elevated in rheumatoid arthritis (Katsikis *et al.* 1994) and that its inhibition by anti-IL-10 antibody leads to increases in active TNF- α and a potential exacerbation of the inflammation. Thus it is possible that IL-10 has a beneficial effect in inflammatory disease. Indeed, a recent preliminary study by Donnelly and co-workers (1995) found that elevated IL-10 in ARDS patients correlated with survival. Hence it may be the case in this study that patients with low levels of IL-10 had less control of their inflammatory response to the trauma they endured and were therefore more likely to develop ARDS. This work is very preliminary however, and a more thorough understanding of the time course of TNF- α and IL-10 release by AMs in ARDS may help unravel their true relevance to the pathophysiology of ARDS and pulmonary inflammation in general.

Soluble TNF receptor is also considered to be anti-inflammatory, since it can inhibit the effects of TNF- α by binding to it in biological fluids (Haran *et al.* 1991). There has been some debate as to whether TNF-R can act as a carrier for TNF- α , so permitting a circulating pool of TNF- α which is resistant to degradation. There is a general consensus, however, that its main role is that of a biological inhibitor (Engelmann *et al.* 1990; Foley *et al.* 1990). TNF-R has been found to be elevated in a number of diseases, including rheumatoid arthritis (Heilig *et al.* 1992; Heilig *et al.* 1993) and sepsis syndrome (Froon *et al.* 1994). Elevated TNF-R has also been measured in human studies following infusion of endotoxin (Shapiro *et al.* 1993; Kuhns *et al.* 1995). This study has demonstrated a substantial elevation in plasma and BALF levels of TNF-R in both patient groups; a substantial elevation in the former and a more moderate increase in the latter. TNF-R was comparable in both ARDS and at risk subjects. We failed to find TNF-R in any AM and PBM supernatants from the two groups however. One explanation for this is that BALF TNF-R is derived from the circulation and arrives in the lung via capillary leak, but this is a less likely explanation for the at risk subjects who have little or no lung injury. Another explanation is that the TNF-R detected is not derived from mononuclear cells. It has been reported that the p55 receptor we measured is more abundant on cells epithelioid in nature (Paleolog *et al.* 1994) which supports an epithelial cell or endothelial cell origin. The dominant form of TNF-R on monocytic cells is the p75 receptor (Leeuwenberg *et al.* 1994), which has not been measured in this study due to the unavailability of a sensitive antibody. TNF-R has also been shown to be shed by T cells (Tartaglia *et al.* 1993), and neutrophils (Porteau and Hieblot, 1994) and their potential as the source cannot be ruled out, although p75 TNF-R is the dominant form of receptor on these cells also. Another explanation which must be considered is that the AMs and PBMs have been stimulated prior to collection by the acute disease process, and their capacity to shed additional receptor in culture may be diminished. Also cell damage cannot be eliminated, especially in the at risk subjects, since their cells failed to produce significant levels of TNF- α , TNF-R or IL-10. However, viability was measured and found to exceed 90% in all cases.

Despite the elevated TNF-R in ARDS it does need to be seen in comparison with the at risk data in order to determine whether this and or the cytokines IL-10 and TNF- α are factors in the progression to ARDS in patients who are at risk. In this study TNF-R was

increased equally in both groups, suggesting a role in trauma and shock, but it is not a prognostic indicator for ARDS. The results for IL-10 and TNF- α however do highlight subtle differences. The ratio of IL-10 to TNF- α is much greater in at risk subjects when compared to ARDS subjects. This could be indicative of inappropriate TNF- α regulation and may be an important mechanism in the pathophysiology of ARDS.

7. ALVEOLAR MACROPHAGE POPULATIONS IN INFLAMMATORY LUNG DISEASE

7.1 Introduction

Alveolar macrophage populations have often been considered to be composed of a homogeneous population of cells, and indeed earlier chapters of this thesis have referred to the AM as if it were a single cell species. However, it has become apparent in recent years that AMs are in fact a heterogeneous population of macrophage-like cells (Poulter, 1983) which can be separated into sub-populations on the basis of cell density (Ferro *et al.* 1987; Fuchs *et al.* 1988) and phenotypic markers (Poulter, 1990).

There are approximately 10^9 resident AMs within the alveoli of the adult human lung, with at least one AM patrolling each alveolus. Although they have a generous life-span of at least several months, they do need to be replenished from time to time, or increased in number, especially during infection or an inflammatory response. Macrophages are derived from the myeloid lineage of haemopoietic cells, and the circulating PBM can be considered the penultimate stage of macrophage differentiation (Stites and Terr, 1987). It is thought that PBMs migrate from the circulation, across the vascular endothelium and the interstitium into the alveolar space where they undergo a final maturation into AMs (Thepen *et al.* 1994). This process is highlighted in the neonate, where at birth the human lung contains few, if any AMs, and over the first few days *post partum* the AM populations are established from circulating PBMs (Fry *et al.* 1984). Monocyte maturation is thought to account for a large proportion of the on-going replenishment of AMs, although some studies have suggested that AMs may proliferate in the lung (Nakata *et al.* 1991). It was presumed that macrophages were terminally differentiated and had therefore lost the capacity for cell division, but it has recently been demonstrated that the human AMs can proliferate, at least *in vitro*, in response to the cytokine GM-CSF (Nakata *et al.* 1991). This mechanism is thought to be important in smokers and subjects with sarcoidosis, where GM-CSF levels in the lung are significantly elevated

(Itoh *et al.* 1993). Thus it seems likely that AM heterogeneity may arise from environmental factors in the lung which may impinge on the newly-recruited PBMs or the established AM populations.

AM population studies to date can be divided into two main areas of research. Some groups have performed density fractionation of AMs using percoll gradients and have noticed that different fractions possess differing functional capacities. For instance IL-1- β secretion and microbicidal activity is found to be greatest in the most dense sub-fraction (Fuchs *et al.* 1988). It has also been postulated that the ability of AMs to suppress T cell activation (accessory cell function) is directly proportional to cell size (Holt *et al.* 1993). The AM increases in size, and thus becomes less dense as it matures from a monocyte to a tissue macrophage, so such studies suggest an increased potency in immature macrophages. This is underlined by studies directly comparing mediator release by AMs and PBMs. IL-1 and IL-6 for example are released in higher quantities by PBMs than AMs, although the opposite is true for TNF (Strieter *et al.* 1989b) and the anti-inflammatory prostanoid PGE₂ (Hempel *et al.* 1994). This is appropriate since during an inflammatory event the resident population is likely to be enriched by extravasation of large numbers of circulating PBMs, thus the balance of established AMs to new recruits may be important in inflammatory lung disease.

Other groups, notably Dr Len Poulter and colleagues at the Royal Free Hospital in London have focused not on cell density, but on the expression of phenotypic markers. These markers have been designated the prefix RFD and are defined in the table below:

Table 7.1

MARKER MOL. WT.		LOCATION
RFD1	28-33 kDa	EPITOPE OF CLASS II MHC
RFDR1	28-33 kDa	AS ABOVE
RFD7	77 kDa	MATURE TISSUE MACROPHAGES
Janossy <i>et al.</i> 1986		

Using a panel of monoclonal antibodies against the RFD antigens Poulter and co-workers have established three sub-populations of AMs:

Table 7.2

POPULATION	PROPORTION IN NORMAL BALF
RFD1 ⁺ D7 ⁺	70-80%
RFD1 ⁺ D7 ⁻	7-15%
RFD1 ⁻ D7 ⁻	4-8%

Functional studies using the autologous mixed lymphocyte reaction (MLR) have determined that RFD1⁺D7⁻ cells are potent APCs, expressing strongly class II MHC with a morphology highly suggestive of DCs (Poulter, 1990; Spiteri and Poulter, 1991). However, co-culture with RFD1⁺D7⁺ AMs can inhibit this accessory cell function (despite their possession of class II MHC), and these cells have been designated suppressor macrophages (Poulter, 1990). RFD1⁺D7⁺ cells form the majority of AMs in the normal lung and are poor accessory cells. However, functional studies with latex beads have demonstrated potent phagocytic capability which is lacking in the other two populations, and the latter are also deficient in lysosomal enzymes required for phagolysosome digestion (Spiteri and Poulter, 1991).

The partial characterisation of these populations has generated some interest in their role in inflammatory lung disease. The suppressive role of AMs has been reported in previous studies (Bilyk and Holt, 1993; Holt *et al.* 1993), and is believed to be important in the prevention of inappropriate inflammatory responses in an environment which is vulnerable to foreign particle bombardment. Indeed, AM depletion in the rat lung has demonstrated that accessory cell function of the remaining DCs in the alveoli, interstitium and BALF is greatly enhanced (Holt *et al.* 1993). Studies with RFD monoclonal antibodies appear to suggest that suppressor function is a property of

RFD1⁺D7⁺ AMs and therefore its distribution may be important in inflammatory disease. It has been shown that in normal gut mucosa these cells represent more than eighty per cent of the macrophage population, but that in the inflammatory bowel condition, Crohn's disease, this is greatly reduced (Allison and Poulter, 1991). In contrast this population represents less than seven per cent of the total AMs in the human lung and appears to increase in sarcoidosis and fibrosing alveolitis (Campbell *et al.* 1986; Poulter, 1990) which are both assumed to have an inflammatory component in their pathogenesis. It seems possible that the balance of RFD1⁺D7⁺ cells is more likely to be implicated in the pathogenesis of interstitial lung disease.

This thesis is based upon the hypothesis that cytokine release (notably TNF- α and IL-10) from AMs has a role in the pathology of inflammatory lung disease. Knowing that AMs are a heterogeneous population of cells the following questions were addressed: (1) is the distribution of AM populations distorted in sarcoidosis, CFA and ARDS?; (2) do AM phenotypes from different diseases correspond in terms of cytokine profile? (3) can AM populations be generated *in vitro* to allow more thorough investigation of their functional properties? AMs from patients and normal subjects were isolated from BALF and analysed by immunocytochemistry for RFD markers. AMs from normal subjects were also purified on the basis of phenotype and cultured for the presence of TNF- α . PBMs from normal subjects and the cell lines THP-1 and U937 were cultured *in vitro* to generate the expression of RFD markers.

7.2 Experimental Design

7.2.1 Phenotypic distribution in normal BALF and the BALF of subjects with lung disease. The ability of the sub-populations to produce TNF- α .

AMs were isolated from BALF. RFD1⁺D7⁺ cells were purified from the non-adherent BAL cells by using negative selection with magnetic beads conjugated with anti-CD3 and anti-CD66b to remove contaminating T cells and PMNs. The other two populations were separated following a two hour adherence by density centrifugation on a metrizamide gradient (refer to Chapter 2). Cytospins of the enriched populations were taken to determine their purity and their intra-cellular expression of TNF- α . To assess distribution of RFD antigens cytopins of total AMs were taken and analysed by double-labelled immunofluorescence (see Chapter 2). CD68 staining was also carried out to confirm macrophage identification. Purified AM subsets were isolated from normal BALF and cultured in RPMI for 24 hours to assess their ability to produce TNF- α . This was determined by ELISA (see Chapter 2).

7.2.2 Induction of RFD1 and RFD7 expression in MDMs, THP-1 and U937 cells

PBMs from normal volunteers were purified by density centrifugation and adherence. They were then cultured for up to twelve days in RPMI + 10% FCS alone, or in the presence of varying concentrations of LPS, PMA, IFN- γ and dexamethasone (dex), separately or in combination. PBMs were cultured at 1×10^6 cells per ml in 24 well tissue culture plates, and cytopins were taken at daily or 2 or 3 day intervals during the culture period. Cytospins were stained for their expression of RFD1 and RFD7 by double-labelled immunofluorescence. THP-1 and U937 cell lines were similarly cultured, either undifferentiated, or in the presence of the maturation factors IL-1- α , PMA, retinoic acid, vitamin D₃ and dibutyryl cAMP. Cytospins were taken and analysed as above.

7.3 Results

7.3.1 Distribution of RFD antigens in normal BALF

Four AM sub-populations could be determined from normal BALF. The major population, accounting for $76.77 \pm 4.18\%$ of the total AMs was the RFD1⁺D7⁺ “mature tissue macrophage” (Figure 7.1). “Accessory” AMs, RFD1⁺D7⁻ accounted for $9.15 \pm 2.93\%$ and the RFD1⁺D7⁺ “suppressor” AMs represented $6.54 \pm 2.77\%$ of the total. $8.08 \pm 1.96\%$ was made up of AMs which were weakly CD68 positive but which did not express RFD antigens.

7.3.2 Distribution of RFD antigens in sarcoidosis

AM populations were distorted in sarcoidosis BALF. Although the major population remained the RFD1⁺D7⁺ phenotype they only represented $34.52 \pm 17.19\%$ of the total, with $29.5 \pm 12.39\%$ RFD1⁺D7⁺ suppressor AMs and $26.25 \pm 10.28\%$ RFD1⁺D7⁻ accessory AMs (Figure 7.2). The accessory and suppressor phenotypes were both significantly elevated when compared to normal subjects ($p < 0.05$). AMs lacking RFD antigens were present in similar proportion to normal BALF, representing $10.75 \pm 2.65\%$ of the total AMs.

7.3.3 Distribution of RFD antigens in CFA

The BALF of CFA subjects was the most distorted of all groups studied. The major population comprised of RFD1⁺D7⁺ suppressor AMs with $49.03 \pm 20.02\%$ of the total (Figure 7.3). This was significantly higher than normal subjects ($p < 0.05$). Likewise RFD1⁺D7⁻ cells were also significantly increased to $29.78 \pm 11.88\%$ ($p < 0.05$). In contrast, the RFD1⁺D7⁺ phenotype was significantly lower ($p < 0.05$) representing only

Figure 7.1 Distribution of RFD1 and RFD7 in AMs derived from normal subjects (n = 16)

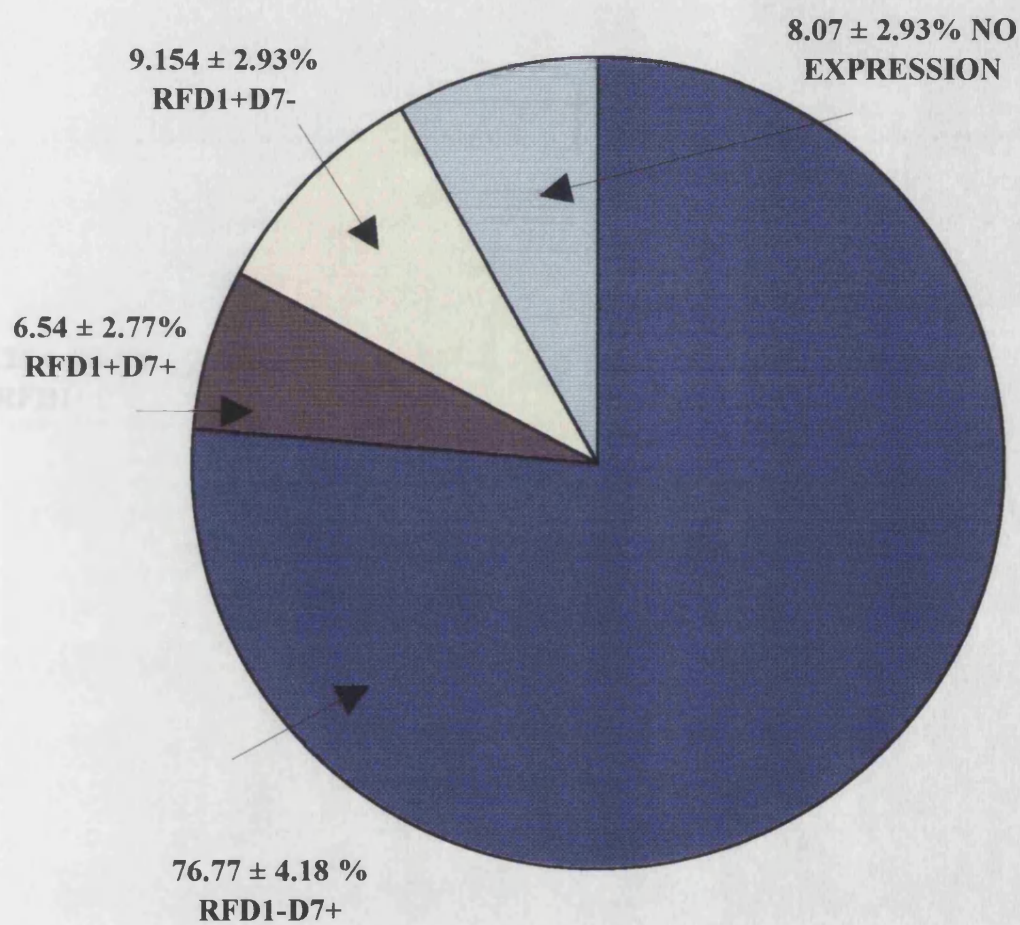


Figure 7.2 Distribution of RFD1 and RFD7 in AMs derived from subjects with sarcoidosis (n=14)

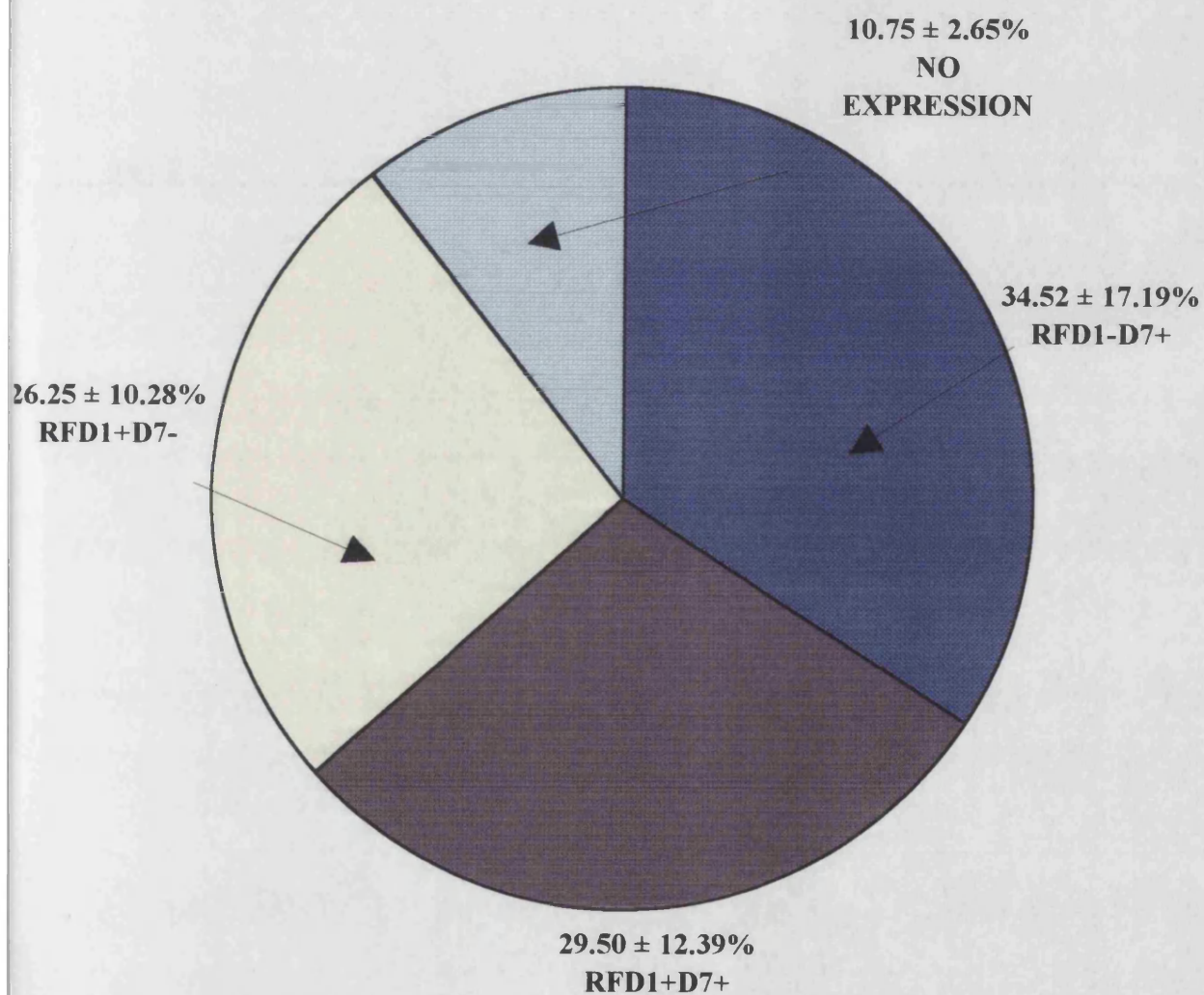
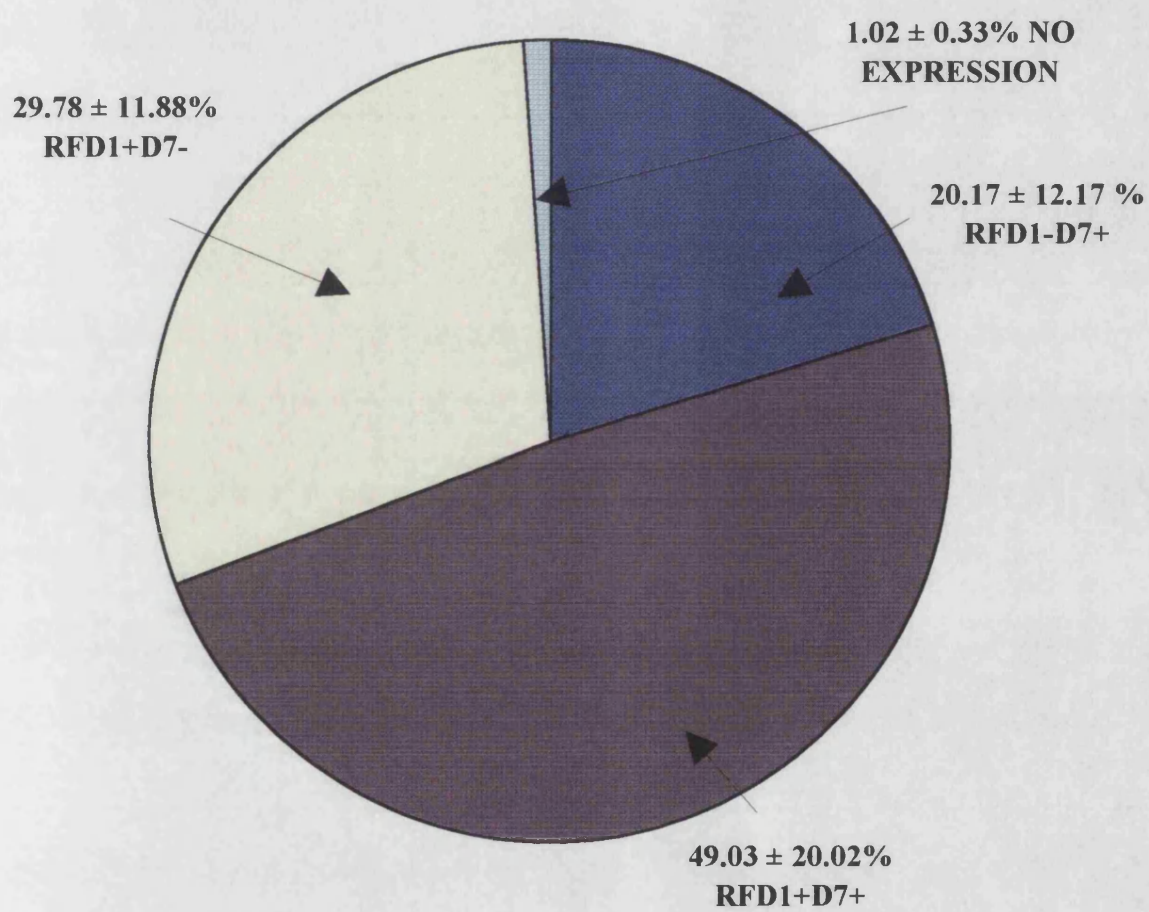


Figure 7.3 Distribution of RFD1 and RFD7 in AMs derived from subjects with CFA (n = 14)



20.17 ± 12.17% of total AMs. Interestingly AMs with no RFD expression formed a very low proportion in CFA BALF, with only 1.02 ± 0.33% ($P < 0.01$).

7.3.4 Distribution of RFD antigens in subjects at risk of ARDS

This group was studied in order to consider any effect of ventilation which may have distorted the profile for ARDS subjects. The population profile of at risk BALF was no different from that seen in normal subjects (Figure 7.4). The distribution was 81.11 ± 4.84% RFD1⁺D7⁺ AMs, 8.33 ± 6.01% RFD1⁺D7⁺ suppressor AMs and 3.89 ± 2.00% RFD1⁺D7⁻ accessory AMs. 6.67 ± 3.65% of AMs had no RFD expression.

7.3.5 Distribution of RFD antigens in subjects with ARDS

RFD1⁺D7⁺ AMs formed the major population in ARDS BALF, representing 67.75 ± 9.68% of the total (Figure 7.5). Expression of RFD1⁺D7⁺ suppressor AMs and RFD1⁺D7⁻ accessory AMs was elevated, although not significantly ($p = 0.058$), when compared to at risk BALF, with 19.75 ± 10.75% and 9.02 ± 4.2% respectively. Cells expressing neither antigen represented 3.48 ± 2.61% of the total.

7.3.6 Intra-cellular TNF- α production and protein release by AM sub-populations from normal BALF

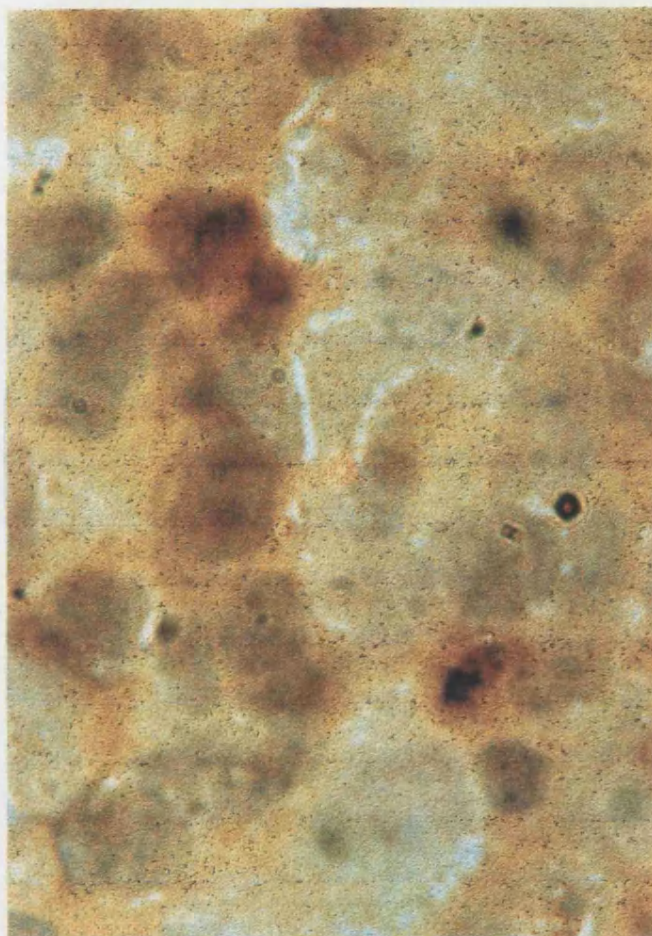
Intra-cellular TNF- α was determined by immunocytochemistry and visualised with DAB substrate (refer to Chapter 2) (see plate 7.1). Although not fully quantitative, relative amounts of TNF- α were expressed as below:

Plate 7.1 TNF- α immunostaining of alveolar macrophages

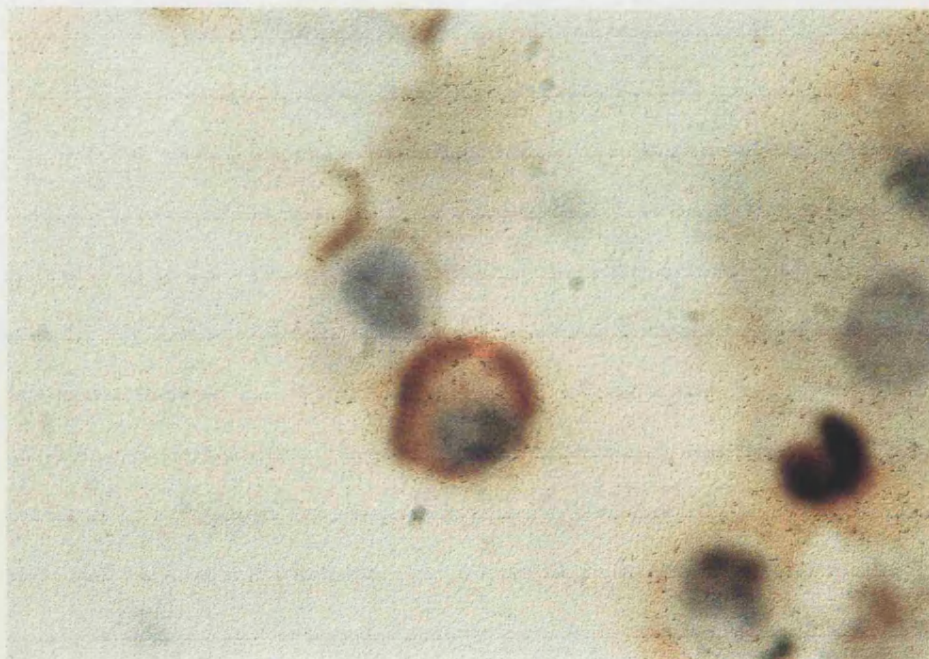
RFD1⁺D7⁻ AMs



RFD1⁺D7⁺ AMs



RFD1⁻D7⁺ AMs



SUBJECT	RFD1 ⁺ D7 ⁻	RFD1 ⁻ D7 ⁺	RFD1 ⁺ D7 ⁺
1	0	+	+
2	0	+	+
3	0	+	+
4	0	++	+++
5	0	++	+++

0 = NO STAINING; + = WEAK; ++ = MODERATE; +++ = STRONG

The ELISA data demonstrated that the RFD1⁺D7⁻ accessory macrophages were unable to produce TNF- α (Figure 7.6). The RFD1⁻D7⁺ AMs were able to elicit a moderate response to LPS, giving 1.31 ± 0.71 ng/ml TNF- α in response to 10 μ g/ml LPS, but there was very little spontaneous TNF- α production (0.103 ± 0.026 ng/ml). In contrast, the RFD1⁺D7⁺ suppressor AMs produced 2.27 ± 0.71 ng/ml spontaneous TNF- α , which rose to 6.79 ± 3.48 ng/ml in response to 10 μ g/ml LPS.

7.3.7 Induction of RFD1 and RFD7 in cultured, unstimulated monocyte-derived macrophages

PBMs were cultured for a total of eleven days without media change. Maturity of MDMs as assessed by CD68 expression was achieved by 5 days in culture (Figure 7.7). RFD1 expression was first observed at day 2, peaking at day 4 with 13% of cells expressing it ($p < 0.05$). By day 10 no RFD1 expression remained. RFD7 expression was delayed until day 3. By day 6 more than 50% of MDMs were expressing RFD7 and this level was maintained throughout the culture period. At no time was dual expression observed.

Figure 7.4 Distribution of RFD1 and RFD7 in AMs derived from subjects at risk of ARDS (n=15)

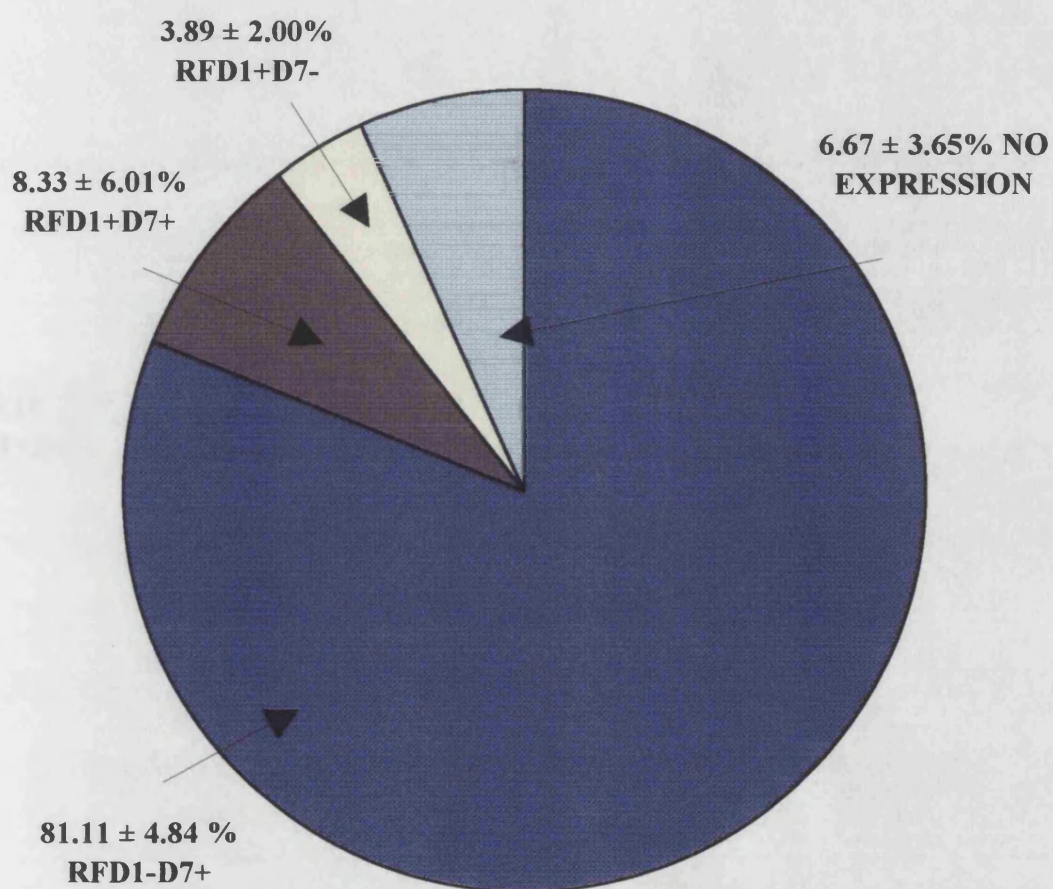


Figure 7.5 Distribution of RFD1 and RFD7 in AMs derived from subjects with ARDS (n=16)

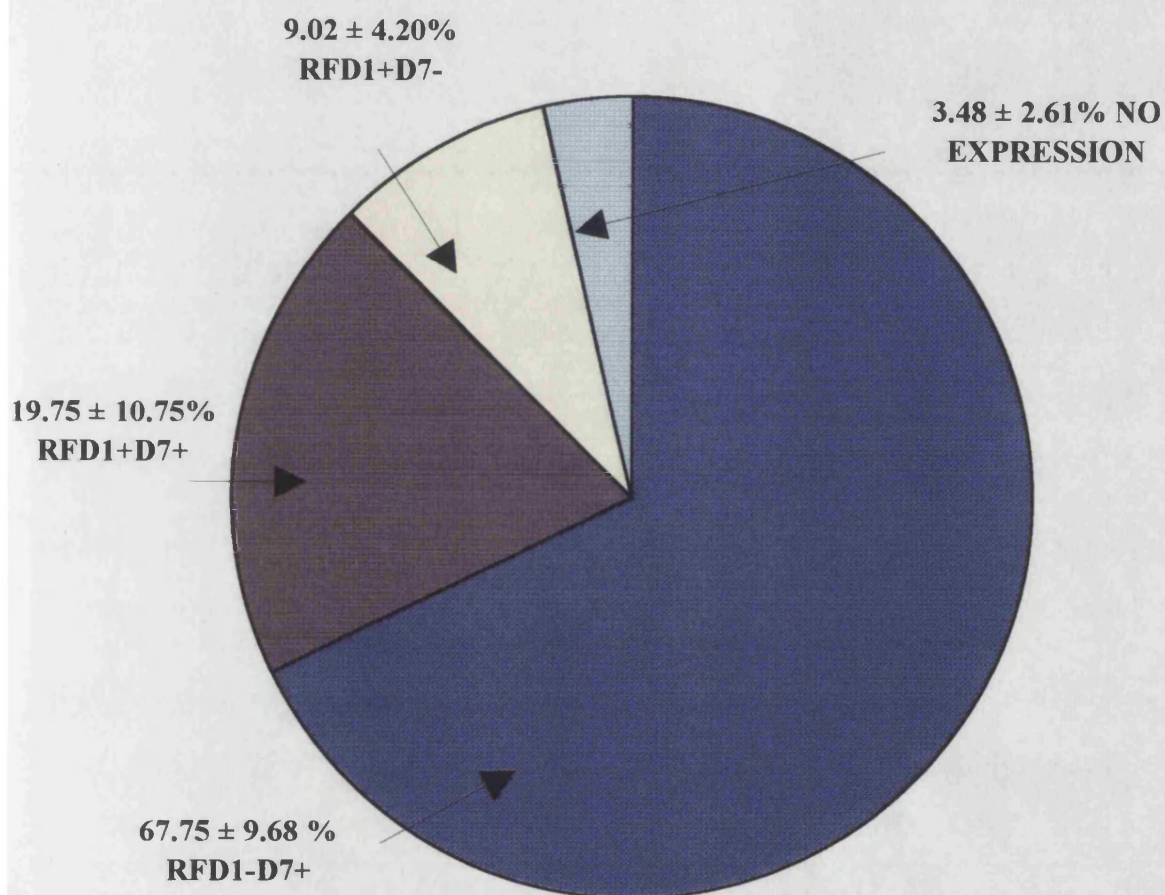
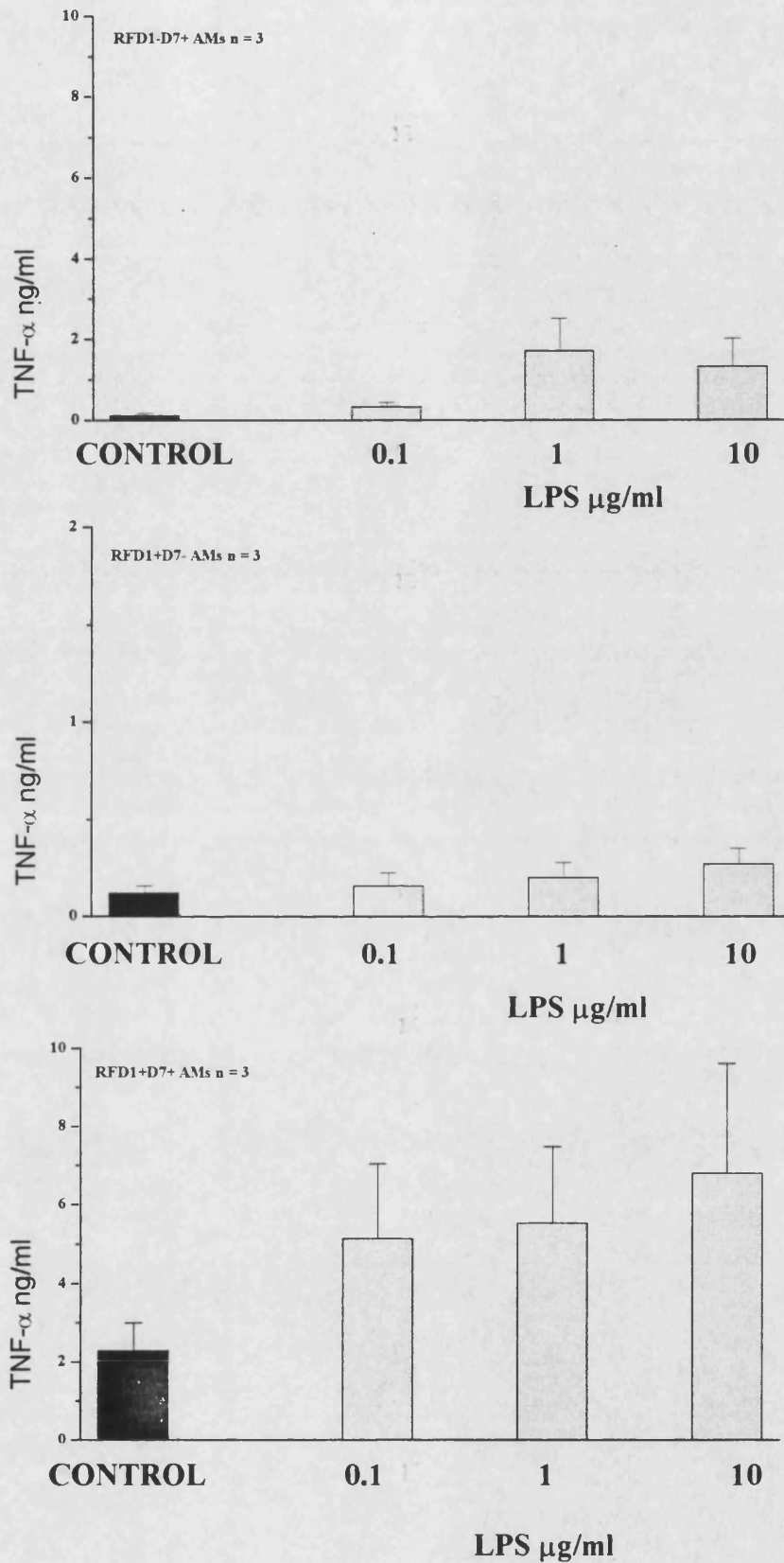
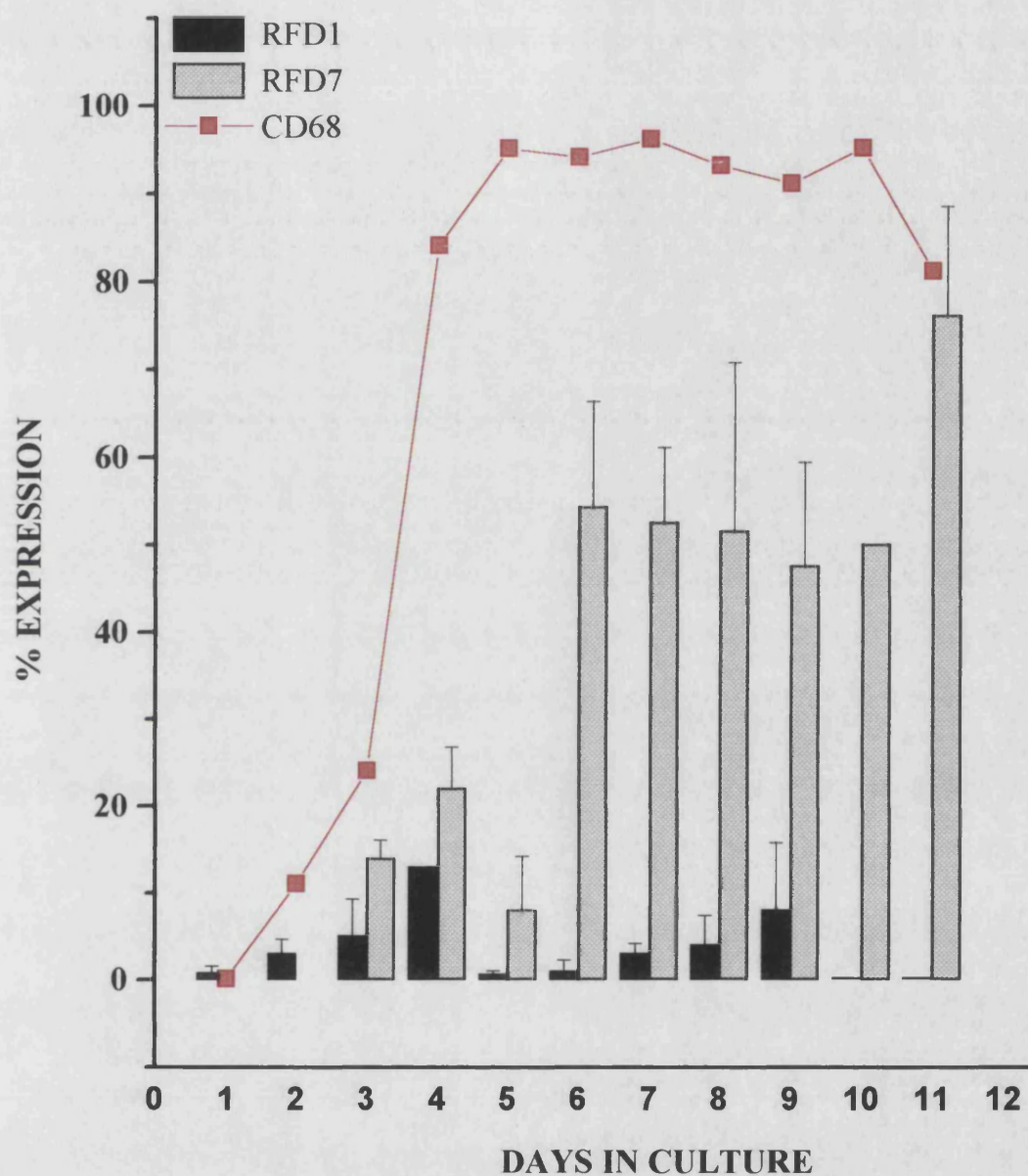


Figure 7.6 SPONTANEOUS AND LPS-INDUCED TNF- α PRODUCTION BY AM SUB-POPULATIONS (n = 3)



Purified populations of AMs cultured for 24 hours, 1×10^6 cells per ml, with a dose range of LPS. Control AMs cultured in medium alone.

Figure 7.7 EXPRESSION OF RFD1 AND RFD7 PHENOTYPIC MARKERS ON PBMS CULTURED IN RPMI + 10% FCS (n = 4)



PBMs from healthy subjects, cultured for up to 11 days, 1×10^6 cells per ml. Cytospins taken at daily intervals to assess expression of RFD markers and CD68 by immunocytochemistry

7.3.8 The effect of LPS and PMA treatment on RFD antigen expression on MDMs

PBMs were isolated and cultured for up to 11 days with 10 µg/ml LPS and 10^{-7} M PMA. Cytospins were taken at intervals and analysed for RFD expression. LPS increased RFD1 expression after 8 days, peaking at day 11 at $31.79 \pm 11.36\%$ whereas RFD7 expression declined after day 8 with only $38.06 \pm 12.01\%$ expression at 11 days (Figure 7.8). In contrast PMA inhibited expression of RFD1 and RFD7 at all time points.

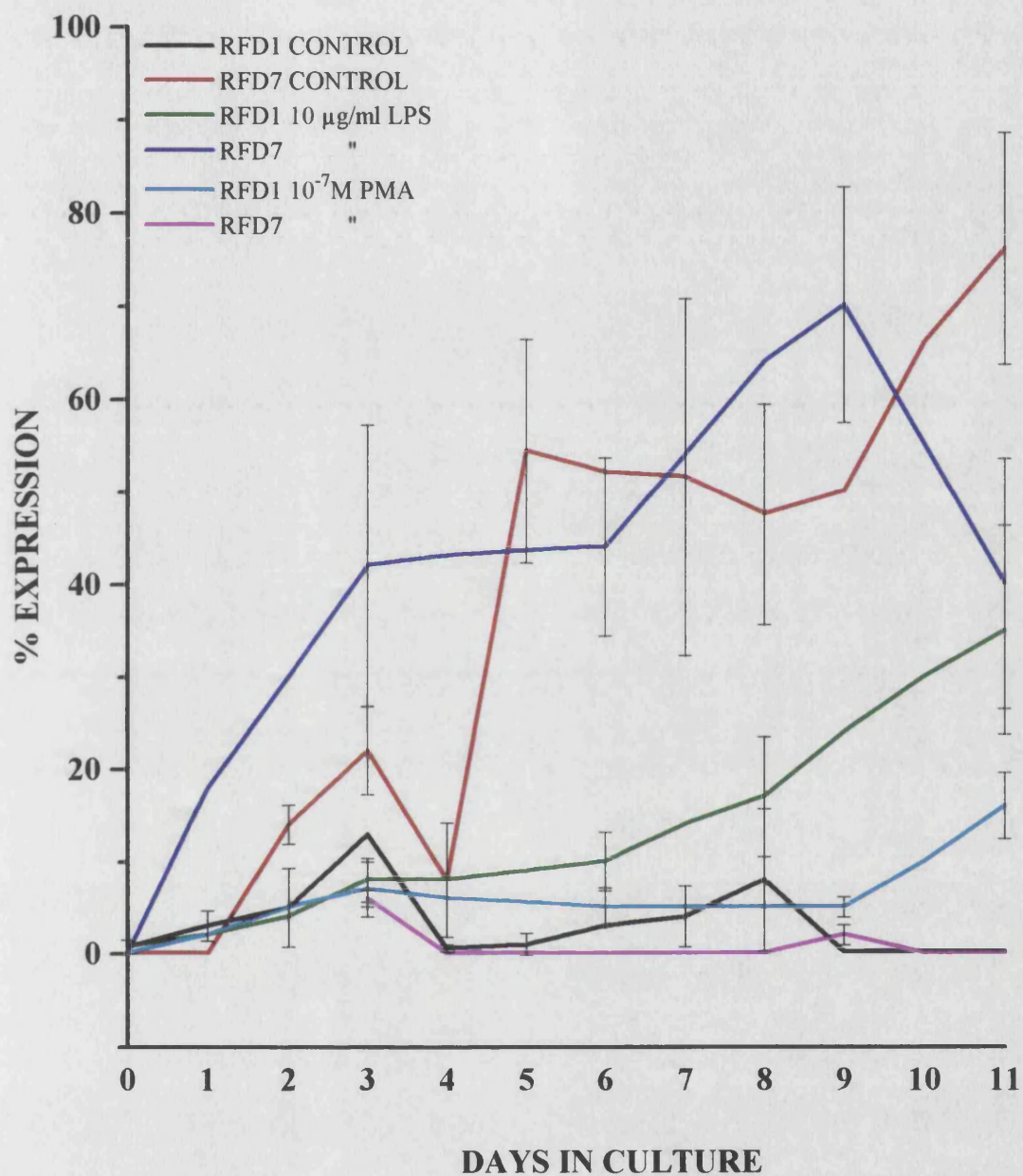
7.3.9 The effect of IFN- γ on RFD expression on MDMs

IFN- γ is known to up-regulate class II MHC expression on monocytes (Trinchieri and Perussia, 1985), and has also been observed to increase RFD1 expression (Dr Len Poulter, personal communication). Various doses of IFN- γ were tried and 250 U/ml was found to be the optimum concentration. In our system IFN- γ did indeed induce RFD1, maximally at day 9, with more than 80% expressing RFD1 ($p < 0.001$) (Figure 7.9). In contrast RFD7 expression was almost totally inhibited at all time points ($p < 0.001$). No dual expression was observed.

7.3.10 The effect of dexamethasone on RFD expression by MDMs

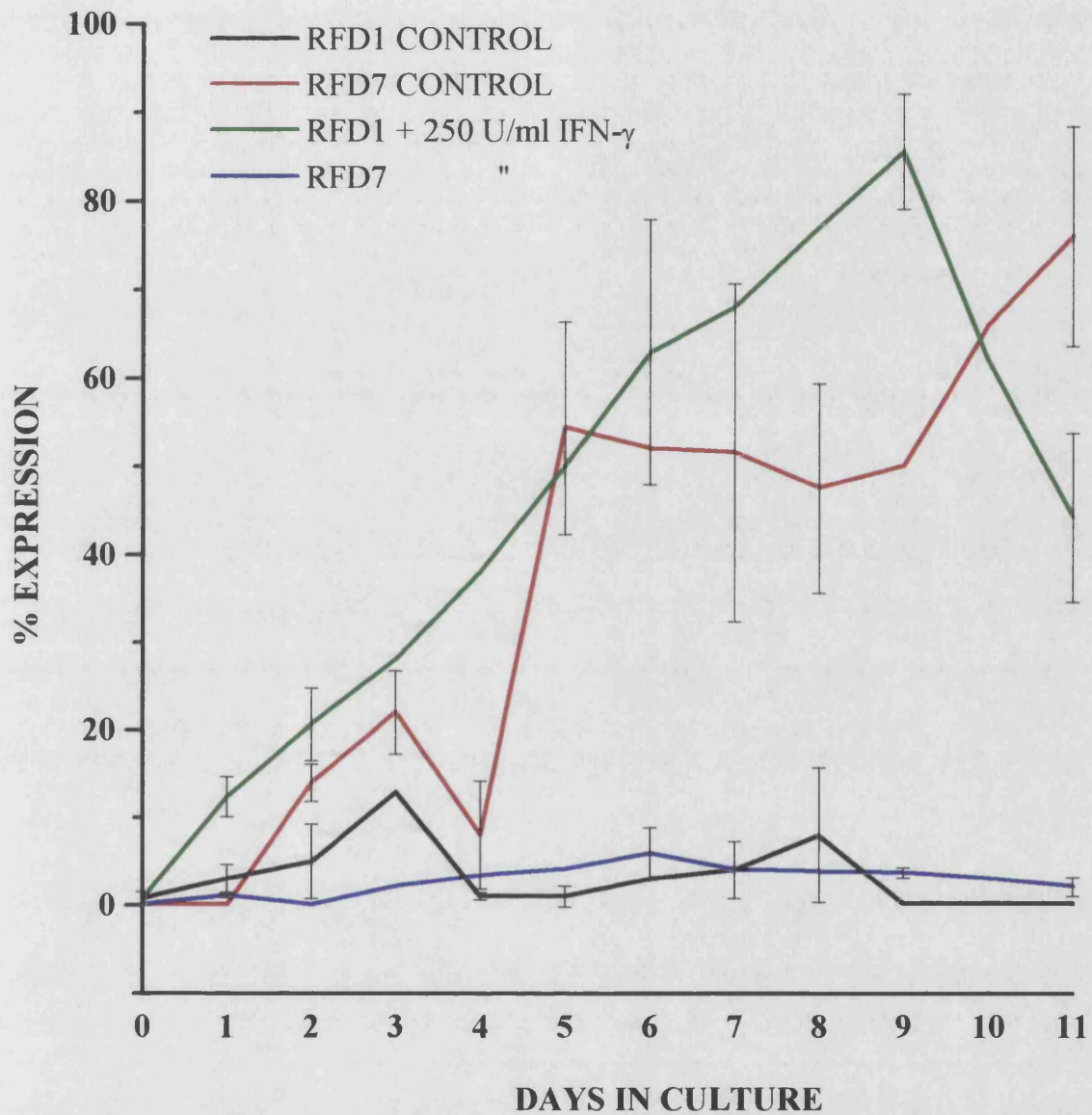
Steroids such as budesonide have been shown to reduce RFD1 expression in diseases such as asthma and CFA (Marianayagam and Poulter, 1991), and Poulter and co-workers have also observed an effect *in vitro* (personal communication). 10^{-7} M dex (optimum concentration) totally inhibited RFD1 expression on MDMs ($p < 0.05$) (Figure 7.10), and in contrast RFD7 was strongly up-regulated to more than 95% positive cells ($p < 0.05$). RFD7 also appeared as early as day 1 in culture and the high level of expression was maintained throughout the culture period. No dual expression was observed.

**Figure 7.8 THE EFFECT OF LPS AND PMA ON RFD1 AND RFD7
EXPRESSION ON PBMs IN CULTURE (n = 4)**



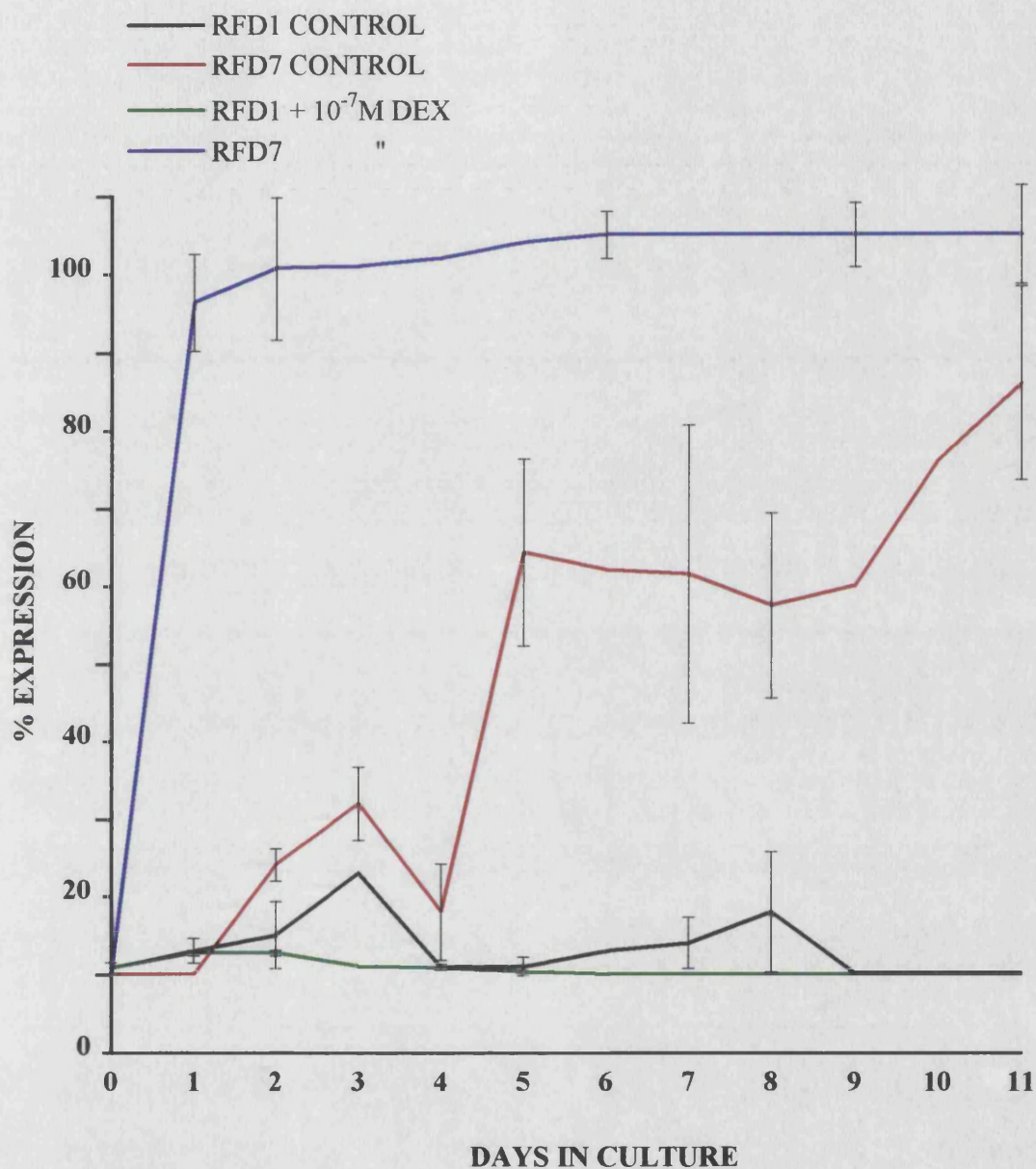
PBMs from healthy subjects, 1×10^6 cells per ml, cultured with or without 10 μ g/ml LPS and 10^{-7} M PMA. Control cells cultured in medium alone

**Figure 7.9 THE EFFECT OF IFN- γ ON RFD1 AND RFD7
EXPRESSION ON CULTURED PBMS (n = 4)**



PBMs from healthy subjects, 1×10^6 cells per ml, cultured with or without 250 U/ml IFN- γ . Control PBMs cultured in medium alone.

**Figure 7.10 THE EFFECT OF DEXAMETHASONE ON RFD1 AND RFD7
EXPRESSION ON CULTURED PBMS (n = 4)**



PBMs from healthy subjects, 1×10^6 cells per ml, cultured with and without 10^{-7} M dexamethasone. Control PBMs cultured in medium containing 0.1% ethanol (vehicle)

7.3.11 The effect of IFN- γ and dex co-culture on RFD expression on MDMs

Failure to induce the RFD1⁺D7⁺ phenotype *in vitro* when using compounds in isolation led to the hypothesis that in combination, IFN- γ and dex may cause a dual up-regulation of RFD1 and RFD7. However this was not achieved in any combination (data not shown).

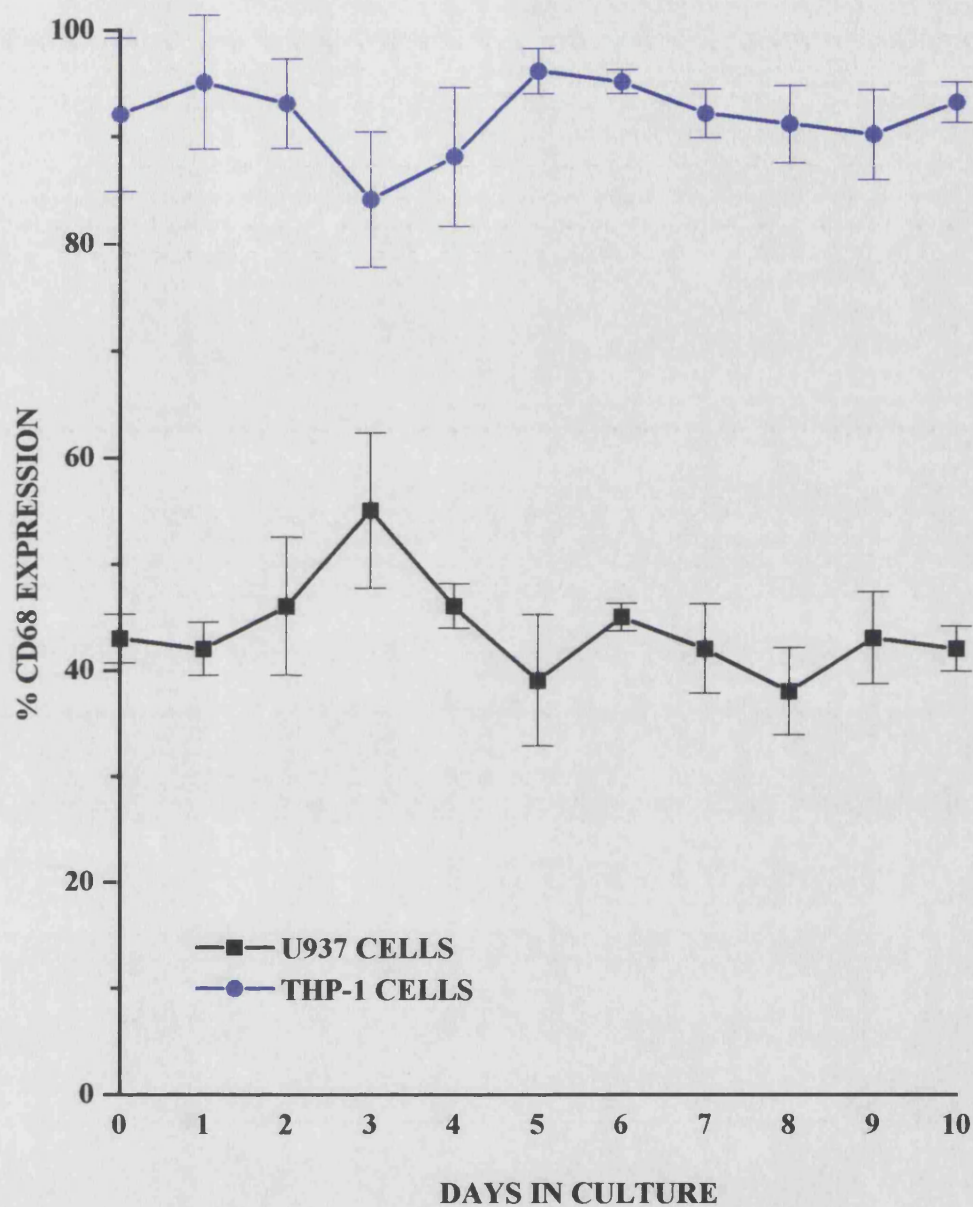
7.3.12 RFD and CD68 expression in undifferentiated U937 and THP-1 cells in culture

U937 and THP-1 cells did not express RFD antigens at any point during a 10 day culture period. THP-1 cells expressed high levels of CD68 expression (in excess of 90%) from day 0, but U937 cells expressed only 45% CD68 maximally throughout culture (Figure 7.11). This suggests that THP-1 cells have a stronger macrophage phenotype than U937 cells.

7.3.13 Induction of RFD antigens in THP-1 cells in response to IL-1- α , retinoic acid (RA) and dibutyryl cAMP

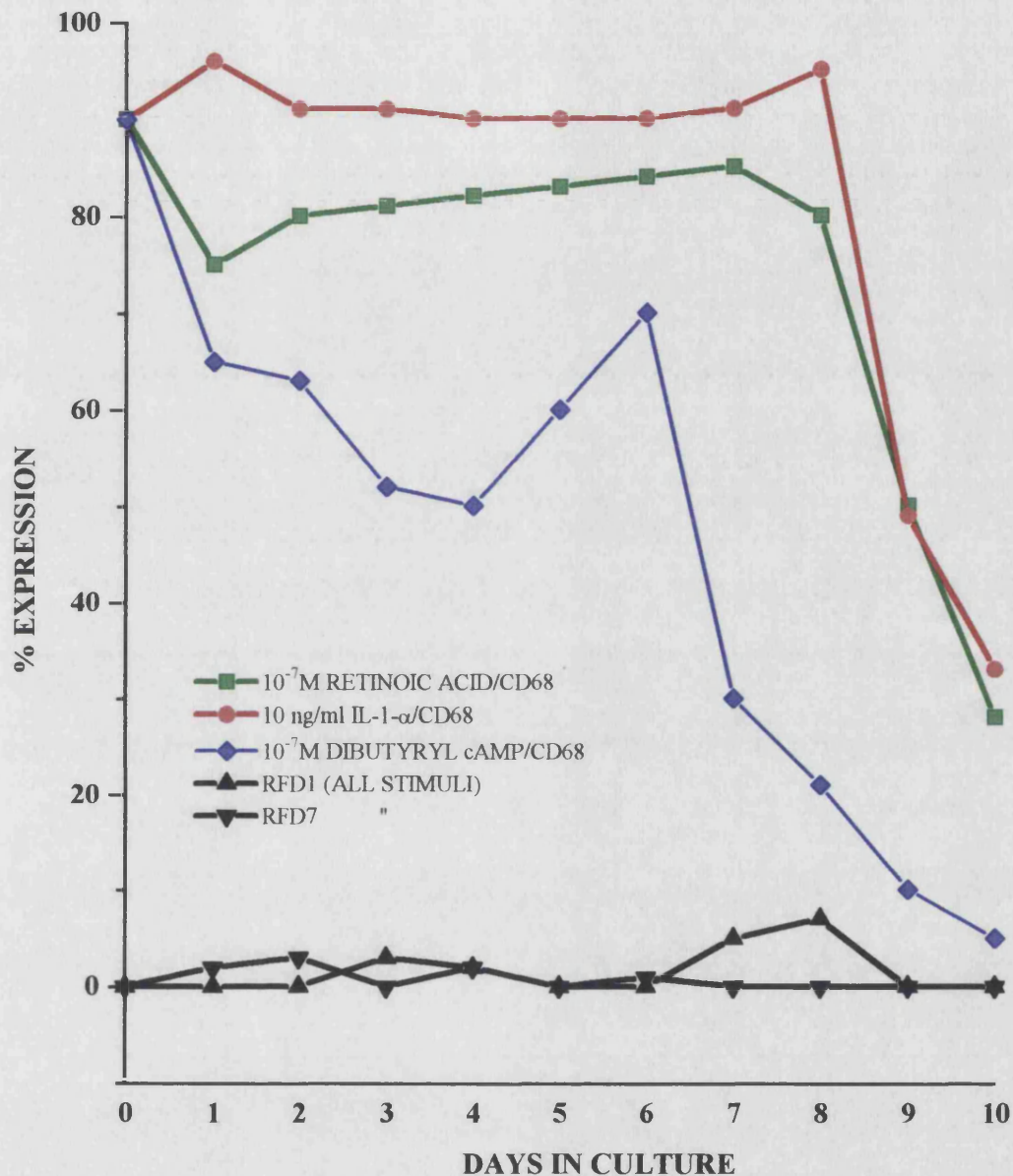
IL-1- α , retinoic acid and dibutyryl cAMP are all recognised inducers of macrophage differentiation in THP-1 cells (Tsuchiya *et al.* 1982). Overnight treatment with 10^{-7} M RA and 10 ng/ml IL-1- α had no recognisable effect on THP-1 cells (Figure 7.12), whereas 10^{-7} M dibutyryl cAMP had the effect of reducing CD68 expression. No changes in morphology were observed following treatment with any compound, indicating that full differentiation had not occurred. There was no RFD antigen induction.

Figure 7.11 THE EXPRESSION OF CD68 ON UNSTIMULATED U937 AND THP-1 CELLS (n = 4)



Ten day culture of U937 and THP-1 cells, 1×10^6 per ml. Cytospins taken at daily intervals for immunocytochemical determination of CD68 expression.

Figure 7.12 THE EFFECT OF RETINOIC ACID, DIBUTYRYL cAMP, AND IL-1 ON MACROPHAGE DIFFERENTIATION IN THP-1 CELLS (n = 3)

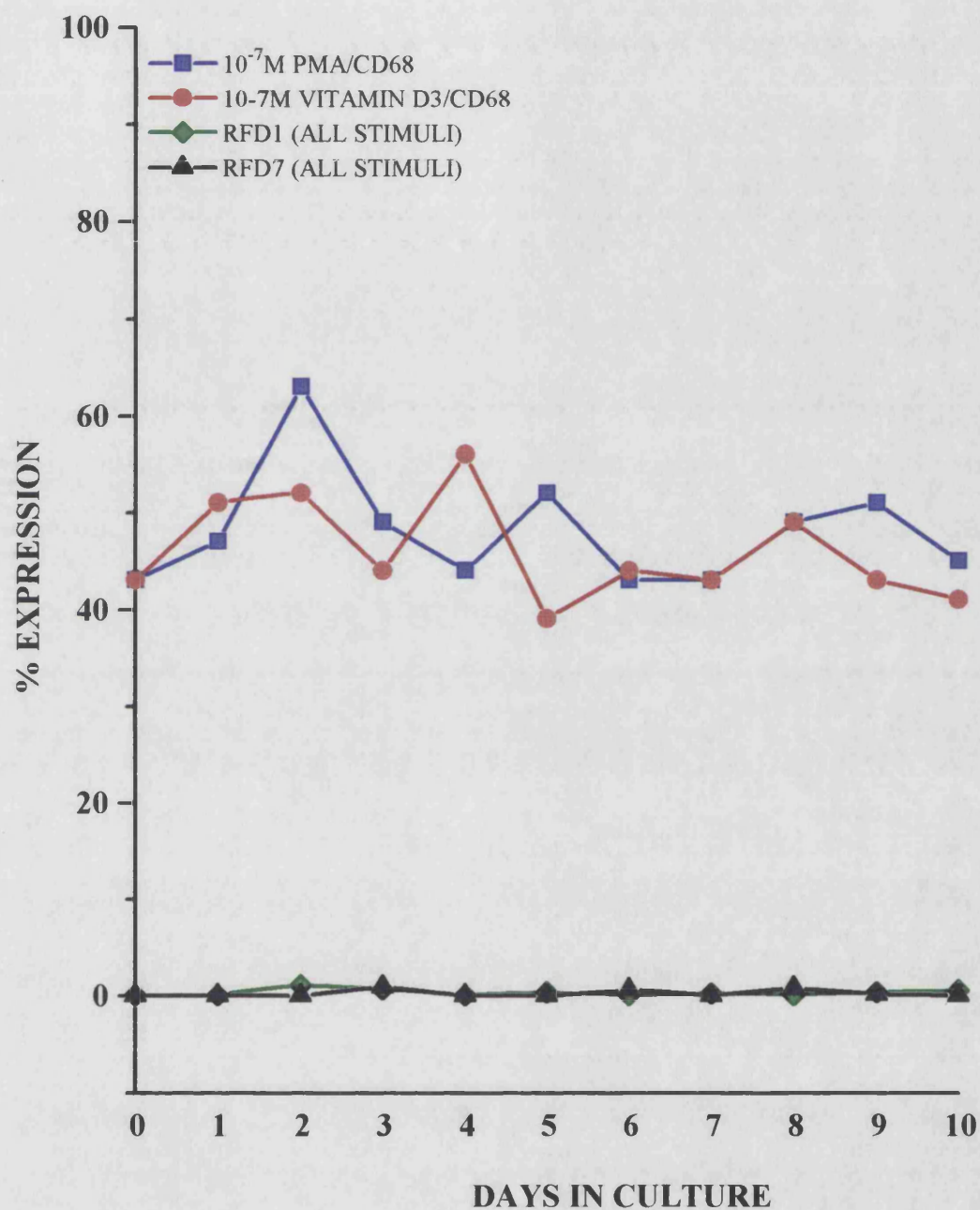


THP-1 cell line, 1×10^6 per ml, cultured with 10^{-7} M retinoic acid, 10 ng/ml IL-1- α or 10^{-7} M dibutyryl cAMP. Control cells cultured in medium alone.

7.3.14 Induction of RFD antigens in U937 cells by PMA and vitamin D₃

PMA, at 10^{-7} M induced a morphological change, inducing adherence and cell proliferation was arrested indicating terminal differentiation. CD68 was upregulated to 83% after 2 days in culture (Figure 7.13), but cells could not be maintained alive for longer than 72 hours following overnight PMA treatment. Vitamin D₃ is a known maturation agent for U937 cells (Amento *et al.* 1984), but 10^{-7} M failed to have any effect on the cells. There was no morphological change or alteration in CD68 expression in response to vitamin D₃. RFD antigen expression was not induced on U937 cells in response to either compound.

**Figure 7.13 THE EFFECT OF PMA AND VITAMIN D₃
ON MACROPHAGE DIFFERENTIATION IN U937 CELLS (n = 3)**



U937 cell line, 1×10^6 cells per ml, cultured with 10^{-7} M PMA or 10^{-7} M vitamin D₃.

7.4 Discussion

The purpose of this study was to examine the distribution of AM sub-populations in the lungs of normal subjects, and the lungs of patients with inflammatory lung disease, and to determine the consequences of any phenotypic variation by measuring the cytokine profiles of AM sub-populations from BALF and phenotypes generated *in vitro*. This would tell us whether a cell exhibiting a specific phenotype e.g. RFD1⁺D7⁺ would exhibit a similar cytokine profile whether it was derived from the lungs of normal subjects and those with inflammatory lung disease, or from MDMs. This would help to address one aspect of the role of sub-populations in the pathogenesis of pulmonary inflammation.

In normal BALF the distribution of AM sub-populations was relatively constant, with the tissue macrophage phenotype of RFD1⁺D7⁺ in the majority. There were relatively low numbers of accessory (RFD1⁺D7⁻) and suppressor (RFD1⁺D7⁺) AMs, as previously reported (Spiteri and Poulter, 1991). Functional studies suggest that such a distribution would be suitable for the normal lung environment, with the emphasis on scavenging and phagocytosis, but with a moderate ability to trigger T cell responses without excessive influence of either accessory or suppressor AMs. Such a balance was also found in subjects with ARDS and at risk of ARDS, (although the former had a slight elevation in the numbers of suppressor macrophages), indicating that phenotype distribution may not be an important element in the pathogenesis of acute lung injury. In BALF from CFA subjects, however, there was a difference, since the highest proportion of AMs were the suppressor macrophages, as previously reported (Noble *et al.* 1989; Campbell *et al.* 1985), but there was also a significantly higher proportion of accessory AMs. Mature tissue macrophages were markedly reduced. This may shift the balance of macrophage activity in the lung away from phagocytosis, but it does not demonstrate whether a cell-mediated inflammatory response is more likely, since accessory AMs are out-numbered by almost 2:1 by suppressor AMs, which have previously been demonstrated to inhibit T cell activation by accessory AMs (Spiteri *et al.* 1992; Spiteri and Poulter, 1991). Sarcoidosis BALF also exhibited elevated populations of AMs expressing the RFD1 antigen, as previously shown (Spiteri *et al.* 1992; Campbell *et al.* 1986; Ainslie *et al.* 1989).

It is difficult to speculate at this time what relevance AM sub-populations may have to the pathogenesis of interstitial lung disease, since functional studies have been limited to accessory cell function and phagocytosis, with no studies as yet on cytokine profiles. This study attempted to examine the TNF- α profiles of the populations so that we may have a preliminary indication of their ability to produce a pro-inflammatory cytokine which is implicated in the pathogenesis of both sarcoidosis and CFA (refer to Chapters' 4 and 5). Isolating the populations for this study was not without its difficulties. The methodology was long-winded, with a number of steps which could have lead to activation or alteration of the cell phenotypes. Also the yield was such that only very low numbers of suppressor and accessory AMs could be purified for culture. Sub-populated AMs were purified from normal BALF and cultured alone and in the presence of LPS. Interestingly the suppressor phenotype produced the most TNF- α , with a moderate yield from RFD1'D7⁺ macrophages and little or no production by accessory AMs. Localisation of TNF- α production to a distinct sub-population has previously been demonstrated in a culture system which allowed determination of TNF- α production by individual AMs (Taylor *et al.* 1993), and showed that TNF- α production in response to LPS was limited to just 20% of the AM population. This thesis has previously demonstrated elevated TNF- α production by AMs in CFA (Chapter 5) and this may now be at least partially explained by the increase in suppressor macrophages in these subjects. Indeed the term "suppressor" AM could be considered a misnomer. The finding that accessory AMs produced barely measurable TNF- α supports the theory that these cells may be closely related to DC, which themselves are very poor producers of soluble mediators (Toews, 1991). Since accessory AMs account for more than 25% of AMs in sarcoidosis, with a ratio of approximately 1:1 to suppressor AMs, compared to 2:1 in CFA, this may help to account for the relatively low production of TNF- α detected in sarcoidosis subjects (refer to Chapter 4), although the adherence purification method used should have eliminated accessory AMs from the culture. Caution must be observed when interpreting this data since cell purity rarely exceeded 80% and contaminating populations was a constant problem. Also AMs in which TNF- α was determined were derived only from normal subjects, so it cannot be automatically assumed that sub-populations from patients with interstitial lung disease would behave in the same manner. An additional problem is the long-winded purification process which could easily distort the behaviour

of the AM sub-populations, either by cell activation or the artificial separation preventing possible cross-talk between the subsets.

One of the greatest problems encountered during this study was low cell numbers. Although a reasonable yield of mature tissue AMs could be purified from normal BALF, only the occasional subject ($n = 4$) yielded enough AMs to purify the other two populations. Although these populations may be obtained more easily from sarcoidosis and CFA subjects, it is not yet known how they may be altered by disease. This led to the pursuit of *in vitro* generation of RFD antigens using MDMs, THP-1 cells and U937 cells. MDM cultures are useful for generating macrophage morphology *in vitro* and are thus widely used as macrophage substitutes (Gessani *et al.* 1993; Elias *et al.* 1985; Mayernik *et al.* 1983). Eleven day culture of MDMs did indeed express the RFD antigens, RFD1 early and RFD7 later, but at no time point could a very high yield of either population be obtained. The use of the class II MHC inducer IFN- γ and the steroid dexamethasone succeeded in generating relatively pure populations of RFD1⁺D7⁻ and RFD1⁻D7⁺ MDMs respectively. However attempts to generate RFD1⁺D7⁺ cells failed. It may be that another cytokine found in the lung environment is required, or the AM close association with DC and epithelial cells in the lung is required for full differentiation. Further attempts to generate the suppressor phenotype in cell lines such as U937 and THP-1 also failed; in fact they could not be stimulated to express any RFD antigens at any stage. This finding stresses the distinction between human cell lines and primary cells in culture and underlines the importance of using fresh human tissue whenever possible in cell biology studies.

The inability to induce all three sub-populations prevented a meaningful functional study of the phenotypes. Although we managed to carry out some very preliminary work measuring TNF- α production, many questions remain. Cytokine production is clearly important in the arena of pulmonary inflammation and it is important to assess how the sub-populations compare to one another in healthy and diseased subjects. One outstanding question is the true relevance of the RFD antigen. RFD7 has been proposed to be a cytoskeletal component, and RFD1 is an epitope of class II MHC, but further characterisation is required. The RFD antigens have been recognised since 1985 and it is somewhat disappointing that no comprehensive characterisation studies have been

performed which may have lead to a CD (cluster of differentiation) classification and a wider body of interest.

8. GENERAL DISCUSSION

The purpose of this thesis was to address the regulation of TNF- α in normal AMs and to determine whether any abnormality in the regulatory mechanisms could be implicated in the pathology of the inflammatory lung diseases sarcoidosis, CFA and ARDS.

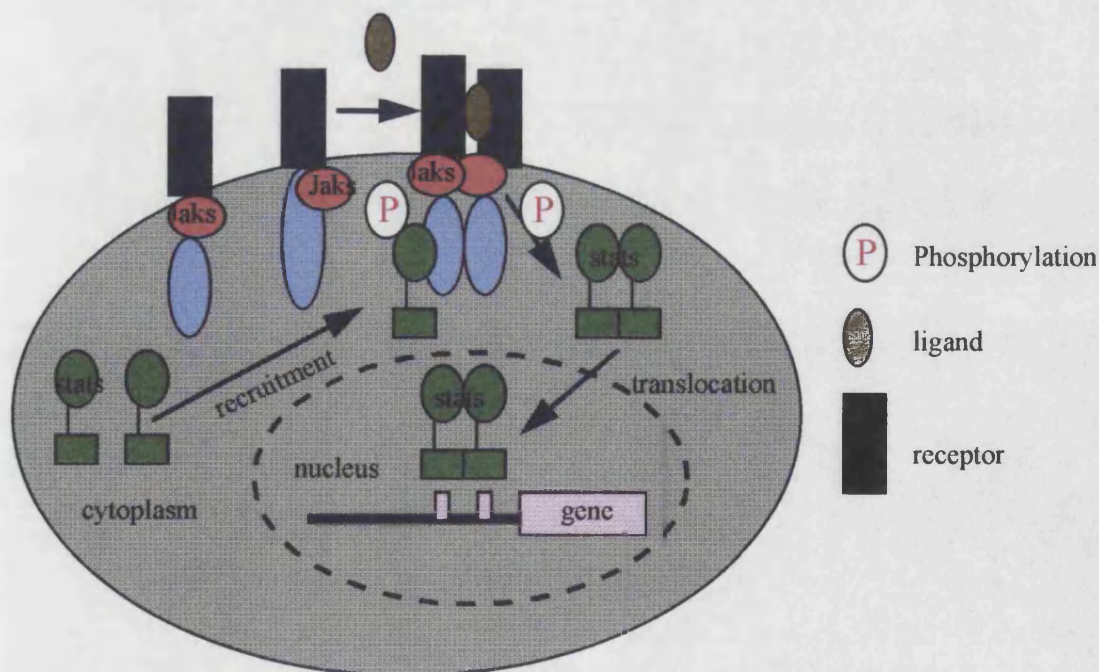
The overall findings of this study have demonstrated that TNF- α protein and mRNA can be inhibited by the addition of IL-10 to human AMs and PBMs in culture, but the mechanisms of this inhibition are not fully understood. In addition, it has highlighted a role for soluble TNF-R as an inhibitor of TNF- α bio-activity. In Chapter 3, northern analysis of AMs and PBMs revealed that TNF- α mRNA expression was reduced in response to IL-10, optimally at 50-100 U/ml (5-10 ng/ml). A decrease in the transcription rate of the TNF- α gene in response to LPS would be an explanation for this, but was not investigated here. To clarify this nuclear run on assays would need to be performed, where cell nuclei are isolated, protein synthesis is arrested, and the formation rate of newly transcribed mRNA is measured using incorporated radio-active nucleotides. Unfortunately, this procedure contains a high degree of difficulty and it takes several months to make the technique reproducible, thus it was not possible to perform nuclear run-on assays in the time-frame of this study. The protein synthesis inhibitor actinomycin was utilised however to ascertain any effect that IL-10 may have on the half-life of TNF- α transcripts. This experiment showed that mRNA degradation was not enhanced by addition of IL-10, an interesting finding, since the TNF- α gene does contain a UA-rich sequence which is thought to confer instability on the resultant transcript. It can therefore be concluded that IL-10 is working at least in part by reducing transcription of the TNF- α gene.

The switching on of gene transcription is a complex process involving the interplay of many intra-cellular messengers. The first step in the process is the binding of the ligand, in this instance IL-10, to its cell surface receptor. Unfortunately, the human IL-10 receptor has as yet not been characterised, and the amino acid sequences of IL-10 which

are critical for binding are unknown. It is well known however that receptor density is an important factor in determining the sensitivity of a cell to the actions of a given cytokine. It is interesting to note that AMs from CFA subjects did show TNF- α inhibition in response to IL-10 at a dose 100 times lower than the minimal response seen in AMs from normal subjects. An increase in IL-10 receptor density could be an explanation for this which is worthy of investigation, but enhanced affinity of the receptor is equally plausible.

Once the ligand has bound to its receptor a cascade of intra-cellular events is initiated, which ultimately leads to the switching on or off of gene transcription. There is currently no published literature on the nature of IL-10 signalling in human cells, but it is known that the receptor belongs to the superfamily of class II cytokine receptors which includes the interferon receptor group (Ihle *et al.* 1996). These receptors have been demonstrated to signal through the JAKs (Janus protein tyrosine kinases) and STATs (signal transduction activated transcription) system as illustrated below:

Figure 8.1 Mechanisms of activation of STATs in signalling by the cytokine receptor superfamily (after Ihle *et al.* 1996)



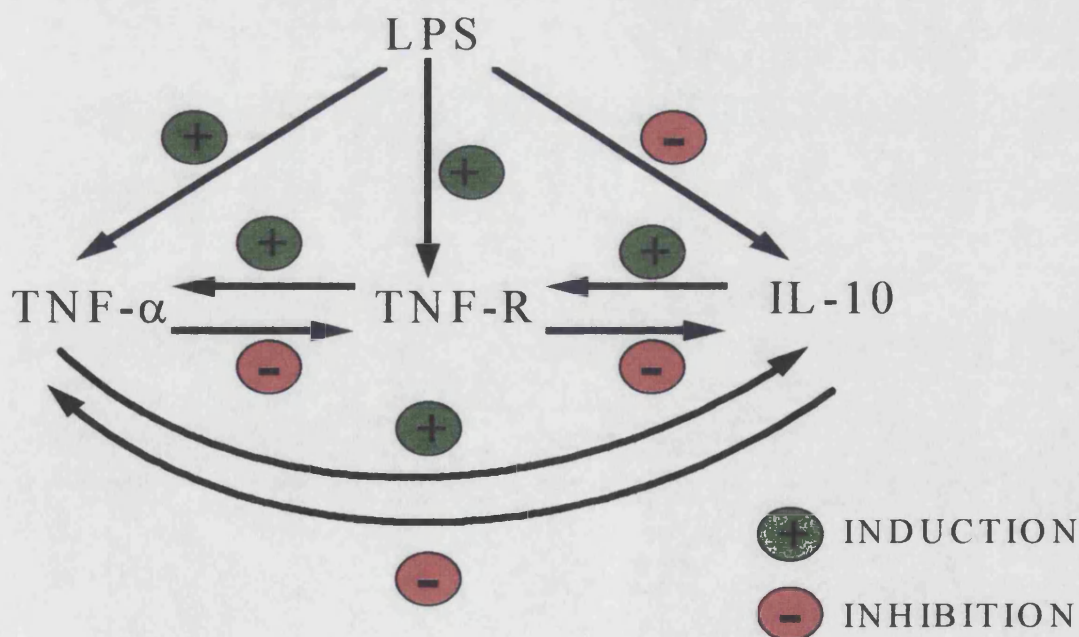
Ligand-induced receptor aggregation initiates a response by bringing the associated JAKs into sufficient proximity to allow trans-phosphorylation and activation of catalytic activity. The JAKs subsequently phosphorylate the receptor chains at multiple sites. STATs are then recruited from the cytoplasm where they interact with tyrosine kinases, resulting in dimerization and translocation into the nucleus. The STAT dimers bind to response elements in the promoter of the cytokine gene, resulting in activation, or in the case of IL-10, inhibition.

This scheme is an over-simplification of events, since there are at least 5 different STAT dimers known, all recognising different sequences in cytokine gene promoters, but it does give an indication of a mechanism by which IL-10 may inhibit TNF- α directly. This mechanism does remain to be confirmed, however and there is strong evidence that LPS-induction of TNF- α in macrophages is dependent upon another type of transcription factor known as nuclear factor kappa B (NF- κ B), which is activated by another intracellular pathway incorporating protein kinase C phosphorylation (Vincenti *et al.* 1992). In this instance NF- κ B dissociates from its cytoplasmic inhibitor I κ B and translocates into the nucleus, where it associates with the appropriate cytokine genes and enhances transcription. There are four known binding sites for NF- κ B on the human TNF- α gene (Vincenti *et al.* 1992). It is not presently known whether IL-10 itself has a direct effect on transcription through the JAK STAT pathway, or whether the effect is indirectly inhibiting the pathway of LPS induction. IL-10 inhibition was not compromised by addition of the transcription inhibitor cyclohexamide thus excluding the requirement for *de novo* protein synthesis for the inhibitory activity of IL-10. This is more indicative of a direct effect. Another line of inquiry which is opened by this study is the role of TNF- α polymorphisms in lung disease. Previous studies have shown the existence of TNF- α variants, which are associated with auto-immunity, and possibly enhanced production of TNF- α (Wilson *et al.* 1993). The most studied variant has an adenine in place of a guanine residue at position 308 in the promoter region of the gene. It would be of great interest to know whether these polymorphisms are regulated differently in response to cytokines such as IL-10 which leads to their pathological effects.

Once these regulatory pathways have been elucidated in normal macrophages it can then be postulated that abnormalities in signalling may account for the heightened sensitivity

to IL-10 in CFA, but in sarcoidosis and ARDS IL-10 inhibition of TNF- α did not appear to be abnormal. Indeed in ARDS patients it was production of IL-10 which appeared to be compromised, which is a process barely touched upon in this thesis. There are no published reports of IL-10 production in alveolar macrophages specifically, but studies have demonstrated an enhancement of IL-10 production by PBMs in response to TNF- α , which is an interesting feedback loop worthy of investigation. This highlights the importance of TNF-R, which in its cell-bound form is the major signalling receptor for TNF- α and therefore its presence on the cell surface could be critical to the amount of IL-10 produced in environment containing TNF- α . In its alternative form, shed from the membrane in response to matrix metalloproteinase enzymes, it is able to inhibit the bioactivity of TNF- α . Either way it seems possible that TNF-R up-regulation may lead to inhibition of TNF- α , either directly by mopping it up in biological fluids, or indirectly by facilitating IL-10 production by macrophages. It is becoming clear that individual cytokines may have limited importance and it is the inter-play of these factors which may result in pathology, as described below:

Figure 8.2 Putative regulatory network for TNF- α in AMs from normal subjects



This scheme represents a simplified view of TNF- α regulation in normal AMs, based on data in this thesis and published findings in human PBMs. It does not indicate a role of IL-13, another regulatory cytokine, since this remains to be established, but even with this omission it is clear that the regulation is complex and there is a great deal of overlap between TNF- α , TNF-R and IL-10. A shift in the balance of this network, for instance, too little TNF-R or IL-10 production, could lead to inappropriate TNF- α bio-activity resulting in a pathological effect. The thesis does propose a role for reduced IL-10 in the pathogenesis of ARDS, as well as demonstrating the masking of immuno-reactive TNF- α by soluble TNF-R in sarcoidosis and CFA, but many questions remain unanswered. This highlights a desirability to examine in some detail TNF- α regulation in a more user-friendly system such as a monocytic/macrophage cell line where limited cell numbers are not an issue and a greater range of experiments could be performed. However it remains the case that human cells, especially those derived from disease subjects do not mirror events seen in cell lines, thus research utilising clinical material is still the most relevant in the arena of biomedical research. Since this is an inescapable fact, methods must be sought to allow a more detailed study of AMs from subjects with inflammatory lung disease.

Isolation of AMs from human subjects has never been a straightforward procedure. Bronchoalveolar lavage is invasive and could lead to activation of AMs during the process of retrieval. Activation as well as cell damage may also occur in the purification process, which in this thesis utilised adherence, followed by cell scraping. There is no easier way of obtaining AMs from human subjects - lung biopsy is even more invasive - but selection by magnetic beads may be a better alternative to adherence purification. This was the protocol of choice in ventilated subjects on ITU since contaminating PMNs in these subjects were often adherent, and it was interesting to note that AMs from subjects at risk of ARDS produce much less TNF- α *in vitro* than normal subjects, perhaps indicating activation of normal AMs prior to culture. Of course, this could also be explained by suppression of AM function *in vivo* by ventilation or the cocktail of drugs administered to ITU patients.

Much of the data in this thesis is of a preliminary nature and increased numbers of patients need to be recruited to establish any strong significance to many of the findings.

There is still much scope also for unraveling the relationship between IL-10, TNF- α and TNF-R in inflammatory lung disease. In terms of the supernatant data obtained, twenty four hours was the chosen time point since this is considered optimum for assessing TNF- α production, but this time point is unlikely to be appropriate for assessing IL-10 and TNF-R production, especially if responding in an autocrine fashion to TNF- α . Further studies with later time points are required to give a more detailed profile analysis of cytokine production in AMs. Unfortunately this does require greater cell numbers. BAL gives a limited return of AMs, usually no more than ten million per subject. The usual cell culture density is one million per ml which does restrict the number of experiments one may carry out on each subject. This difficulty may be solved by adopting a procedure known as ELISPOT, which permits protein determination on only a handful of cells using antibodies added directly to the cell culture. Polymerase chain reaction (PCR) is another technique which would eliminate the requirement for large cell numbers. Northern analysis requires at least five million cells per point to isolate sufficient mRNA for study, but reverse transcriptase PCR, which makes DNA copies of the mRNA and amplifies it many times, requires one hundred thousand cells per point, so permitting a more detailed investigation of cytokine expression and regulation at the molecular level. The detection of TNF-R could also be improved by assaying for p75 receptor as well as p55. The antibody for p75 detection has recently become available, and we have preliminary indications suggesting that it is produced in higher quantities than p55, especially in sarcoidosis and CFA. The IL-10 receptor is also likely to be an important factor, since increased potency of IL-10 inhibition of TNF- α may be explained by IL-10R up-regulation, either spontaneously or in response to LPS. An appropriate antibody is unavailable at this time, but binding studies using labelled IL-10 could give insight into its recognition by AMs. Also, it is not appropriate to concentrate solely on the AM as a source of these proteins. Other cells are undoubtedly indicated, including T and B lymphocytes, PMNs and epithelial cells. Immunocytochemistry and *in situ* PCR of lung biopsy tissue could give insight into the possible sources of TNF- α , IL-10 and TNF-R and explain the finding that although BALF TNF- α is elevated without exception in all three inflammatory lung diseases investigated, there was not always increased TNF- α production by AMs. Finally, it was attempted to purify the AM sub-populations in this study in order to evaluate whether it is appropriate to examine cytokine profiles on a

heterogeneous population of AMs in disease. However, methodological problems compromised this particular study and it was not possible to perform cultures on separated AMs from disease subjects, or even carry out a more detailed study on cytokine profiles in AM sub-populations from the normal lung. Thus it must be understood that the majority of conclusions drawn refer to cytokine release by co-cultured tissue and suppressor macrophages only.

In summary this study has attempted to unravel the nature of the relationship between AM-derived TNF- α and the regulatory proteins IL-10 and TNF-R. In so doing it has raised a multitude of questions and opened up many possible avenues of investigation. By pursuing one or more of these lines of investigation it is hoped that one would be closer to understanding the precise role of TNF- α in the pathogenesis of inflammatory lung disease.

9. BIBLIOGRAPHY

Abrams, J. S., Roncarolo, M., Yssel, H., Andersson, U., Gleich, G. J., and Silver, J. E. Strategies of anti-cytokine monoclonal antibody development: immunoassay of IL-10 and IL-5 clinical samples. *Immunol. Rev.* 127:6-24, 1992.

Aderka, D., Engelmann, H., Maer, Y., Brakebusch, C., and Wallach, D. Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. *J. Exp. Med.* 175:323-329, 1992.

Aggarwal, B. B. and Pocsik, E. Cytokines: from clone to clinic. *Arch. Biochem. Biophys.* 292(2):335-359, 1992.

Ainslie, G. M., Poulter, L. W., and du Bois, R. M. Relation between immunocytological features of bronchoalveolar lavage fluid and clinical indices in sarcoidosis. *Thorax* 44:501-509, 1989.

Allison, M. C. and Poulter, L. W. Changes in phenotypically distinct mucosal macrophage populations may be a prerequisite for the development of inflammatory bowel disease. *Clin. Exp. Immunol.* 85:504-509, 1991.

Amento, E. P., Bhalla, A. K., Kurnick, J. T., Kradin, R. L., Clemens, T. L., Holick, S. A., Holick, M. F., and Krane, S. M. 1a,25-dihydroxy vitamin D₃ induces maturation of the human monocyte cell line U937, and, in association with a factor from human T lymphocytes, augments production of a monokine, mononuclear cell factor. *J. Clin. Invest.* 73:731-739, 1984.

Ashbaugh, D. G., Bigelow, D. B., Petty, T. L., and Levine, B. E. Acute respiratory distress in adults. *Lancet* II:319-323, 1967.

Aversa, G., Punnonen, J., Cocks, B. G., de Waal Malefyt, R., Vega, F. ,Jr., Zurawski, S. M., Zurawski, G., and de Vries, J. E. An interleukin 4 (IL-4) mutant protein inhibits both IL-4 or IL-13-induced human immunoglobulin G4 (IgG4) and IgE synthesis and B cell proliferation: Support for a common component shared by IL-4 and IL-13 receptors. *J. Exp. Med.* 178:2213-2218, 1993.

Bachwich, P. R., Chensue, S. W., Larrick, J. W., and Kunkel, S. L. Tumor necrosis factor stimulates interleukin-1 and prostaglandin E2 production in resting macrophages. *Biochem. Biophys. Res. Commun.* 136:94-101, 1986a.

Bachwich, P. R., Lynch, J. P., and Larrick, J. Tumor necrosis factor production by human sarcoid alveolar macrophages. *Am. J. Pathol.* 125:421-425, 1986b.

Baggiolini, M., Walz, A., and Kunkel, S. L. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J. Clin. Invest.* 84:1045-1049, 1989.

Barzo, P. Familial idiopathic fibrosing alveolitis. *Eur. J. Respir. Dis.* 66:350-352, 1985.

Baughman, R. P., Lower, E. E., Pierson, G., and Strohofer, S. Spontaneous hydrogen peroxide release from alveolar macrophages of patients with active sarcoidosis: comparison with cigarette smokers. *J. Lab. Clin. Med.* 111:399-404, 1988.

Beutler, B., Milsark, I. W., and Cerami, A. C. Passive immunisation against cachectin/tumour necrosis factor protects mice from lethal effect of endotoxin. *Science* 229:869-871, 1985.

Beutler, B., Krochin, N., Milsark, I. W., Luedke, C., and Cerami, A. Control of cachectin (tumor necrosis factor) synthesis: Mechanisms of endotoxin resistance. *Science* 232:977-980, 1986.

Beutler, B., Thompson, P., Keyes, J., Hagerty, K., and Crawford, D. Assay of a ribonuclease that preferentially hydrolyses mRNAs containing cytokine-derived UA-rich instability sequences. *Biochem. Biophys. Res. Commun.* 152:1988.

Beutler, B. and Cerami, A. The biology of cachectin/TNF - primary mediator of the host response. *Annu. Rev. Immunol.* 7:625-655, 1989.

Bevilacqua, M. P., Pober, J. S., Majeau, G. R., Fiers, W., Cotran, R. S., and Gimbrone, M. A. Recombinant tumor necrosis factor-induced procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. *Proc. Natl. Acad. Sci. USA* 83:4533-4537, 1986.

Billiau, A. Gamma-interferon: the match that lights the fire? *Immunol. Today* 9(2):37-40, 1988.

Billiau, A. and Vandekerckhove, F. Cytokines and their interactions with other inflammatory mediators in the pathogenesis of sepsis and septic shock. *Eur. J. Clin. Invest.* 21:559-573, 1991.

Bilyk, N. and Holt, P. G. Inhibition of the immunosuppressive activity of resident pulmonary alveolar macrophages by granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 177:1773-1777, 1993.

Bogdan, C., Paik, J., Vodovotz, Y., and Nathan, C. Contrasting mechanisms for the suppression of macrophage cytokine release by transforming growth factor-beta and Interleukin-10. *J. Biol. Chem.* 267:23301-23308, 1992.

Bogdan, C. and Nathan, C. Modulation of macrophage function by transforming growth factor β , interleukin-4, and interleukin-10. *Ann. NY Acad. Sci.* 685:713-739, 1993.

Bone, R. C. A new therapy for the adult respiratory distress syndrome. *N. Engl. J. Med.* 328(6):431-432, 1993.

Brandt, E., Petersen, F., and Flad, H. Recombinant tumor necrosis factor- α potentiates neutrophil degranulation in response to host defense cytokines neutrophil-activating peptide 2 and IL-8 by modulating intracellular cyclic AMP levels. *J. Immunol.* 149:1356-1364, 1992.

Braude, S., Nolop, K. B., Hughes, J. M. B., Barnes, P. J., and Royston, D. Comparison of lung vascular and epithelial permeability indices in the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 133:1002-1005, 1986.

Brieland, J. K., Flory, C. M., Jones, M. L., Miller, G. R., Remick, D. G., Warren, J. S., and Fantone, J. C. Regulation of monocyte chemoattractant protein-1 gene expression and secretion in rat pulmonary alveolar macrophages by lipopolysaccharide, tumor necrosis factor- α and interleukin-1- β . *Am. J. Respir. Cell Mol. Biol.* 12:104-109, 1995.

Brown, Z., Gerritsen, M. E., Strieter, R. M., Kunkel, S. L., and Westwick, J. Human lung microvascular endothelial cells secrete IL-8 and MCP-1: differential regulation by interferon gamma. *Am. J. Pathol.* 154:1-9, 1993.

Burke-Gaffney, A. and Keenan, A. K. Does TNF- α directly increase endothelial cell monolayer permeability? *Agents & Actions* 38:C83-C85, 1993.

Campa, J. S., Harrison, N. K., and Laurent, G. J. Regulation of matrix production in the airways. In: *T-Lymphocyte and Inflammatory cell Research in Asthma*, edited by G. Jolles, J. Karlsson and J. Taylor. London: Academic Press, 1993, p. 221-239.

Campbell, D. A., Poulter, L. W., Janossy, G., and du Bois, R. M. Immunohistological analysis of lung tissue from patients with cryptogenic fibrosing alveolitis suggesting local expression of immune hypersensitivity. *Thorax* 40:405-411, 1985.

Campbell, D. A., Poulter, L. W., and du Bois, R. M. Phenotypic analysis of alveolar macrophages in normal subjects and in patients with interstitial lung disease. *Thorax* 41:429-434, 1986.

Camussi, G., Albano, E., Tetta, C., and Bussolino, F. The molecular action of tumour necrosis factor alpha. *Eur. J. Biochem.* 202:3-14, 1991.

Cannon, J. G., Tompkins, R. G., Gelfand, J. A., Michie, H. R., Stanford, G. G., van der Meer, J. W. M., Endres, S., Lonnemann, G., Corsetti, J., Chernow, B., Wilmore, D. W., Wolff, S. M., Burke, J. F., and Dinarello, C. A. Circulating interleukin-1 and tumour necrosis factor in septic shock and experimental endotoxin fever. *J. Infect. Dis.* 161:79-84, 1990.

Carré, P. C., Mortenson, R. L., King, T. E., Noble, P. W., Sable, C. L., and Riches, D. W. H. Increased expression of the interleukin-8 gene by alveolar macrophages in idiopathic pulmonary fibrosis. *J. Clin. Invest.* 88:1802-1810, 1991.

Carswell, E., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. An endotoxin-induced serum factor that causes necrosis of tumours. *Proc. Natl. Acad. Sci. USA* 72:3666-3670, 1975.

Cassatella, M. A., Meda, L., Bonora, S., Ceska, M., and Constantin, G. Interleukin 10 (IL-10) inhibits the release of proinflammatory cytokines from human polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1 β in mediating the production of IL-8 triggered by lipopolysaccharide. *J. Exp. Med.* 178:2207-2211, 1993.

Chikanza, I. C., Roux-Lombard, P., Dayer, J., and Panayi, G. S. Tumor necrosis factor soluble receptors behave as acute phase reactants following surgery in patients with rheumatoid arthritis, chronic osteomyelitis and osteoarthritis. *Clin. Exp. Immunol.* 92:19-22, 1993.

Chirgwin, J., Przybyla, A., MacDonald, R., and Rutter, W. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Am. Chem. Soc.* 18:5294-5299, 1979.

Chollet-Martin, S., Montravers, P., Gibert, C., Elbim, C., Desmonts, J. M., Fagon, J. Y., and Gougerot-Pocidallo, M. A. Subpopulation of hyperresponsive polymorphonuclear neutrophils in patients with adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 146:990-996, 1992.

Chollet-Martin, S., Montravers, P., Gibert, C., Elbim, C., Desmonts, J. M., Fagon, J. Y., and Gougerot-Pocadilo, M. A. Relationships between polymorphonuclear neutrophils and cytokines in patients with adult respiratory distress syndrome. *Ann. NY Acad. Sci.* 354-366, 1994.

Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159, 1987.

Cohen, A. B., Macarthur, C., Idell, S., Maunder, R., Martin, T., Dinarello, C. A., Griffith, D., and McLarty, J. A peptide from alveolar macrophages that releases neutrophil enzymes into the lungs in patients with the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 137:1151-1158, 1988.

Cohen, A. B. and Cline, M. J. The human alveolar macrophage: isolation, cultivation in vitro, and studies of morphologic and functional characteristics. *J. Clin. Invest.* 50:1390-1397, 1971.

Corti, A., Fassina, G., Marcucci, F., Barbanti, E., and Cassani, G. Oligomeric tumour necrosis factor α slowly converts into inactive forms at bioactive levels. *Biochem. J.* 284:905-910, 1992.

Costabel, U., Bross, K. J., Ruhle, K. H., Lohr, G. W., and Matthys, H. Ia-like antigens on T-cells and their subpopulations in pulmonary sarcoidosis and hypersensitivity pneumonitis. *Am. Rev. Respir. Dis.* 131:337-342, 1985.

Crystal, R. G., Gadek, J. E., Ferrans, V. J., Fulmer, J. D., Line, B. R., and Hunninghake, G. W. Interstitial lung disease: current concepts of pathogenesis, staging and therapy. *Am. J. Med.* 70:542-568, 1981a.

Crystal, R. G., Roberts, W. C., Hunninghake, G. W., Gadek, J. E., Fulmer, J. D., and Line, B. R. Pulmonary sarcoidosis - a disease characterised and perpetuated by activated lung lymphocytes-T. *Ann. Internal Med.* 94:73-94, 1981b.

Crystal, R. G., Bitterman, P. B., Rennard, S. I., Hance, A. J., and Keogh, B. A. Interstitial lung diseases of unknown cause. *N. Engl. J. Med.* 310:154-166, 1984.

Dalhoff, K., Bohnet, S., Braun, J., Kreft, B., and Wiebmann, K. J. Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of mononuclear cell alveolitis in pulmonary sarcoidosis. *Thorax* 48:1140-1144, 1993.

Damas, P., Reuter, A., Gysen, P., Demonty, J., Lamy, M., and Franchimont, P. Tumour necrosis factor and interleukin-1 serum levels during severe sepsis in humans. *Critical care medicine* 17:975-978, 1989.

Daniele, R. P., Elias, J. A., Epstein, P. E., and Rossman, M. D. Bronchoalveolar lavage: role in the pathogenesis, diagnosis and management of interstitial lung disease. *Ann. Internal Med.* 102:93-108, 1985.

Dannenberg, J. M. J. Macrophages in inflammation and infection. *N. Engl. J. Med.* 293:489-493, 1975.

de Waal Malefyt, R., Abrams, J., Bennet, B., Figdor, C. G., and de Vries, J. E. Interleukin-10 (IL-10) inhibits cytokine synthesis by human monocytes - an autoregulatory role for IL-10 produced by monocytes. *J. Exp. Med.* 174:1991a.

de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M., te Velde, A., Figdor, C., Johnson, K., Yssel, H., and de Vries, J. E. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen specific human T cell proliferation by diminishing the antigen presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J. Exp. Med.* 174:915-924, 1991b.

de Waal Malefyt, R., Figdor, C. G., Huijbens, R., Mohan-Peterson, S., Bennett, B., Culpepper, J., Dang, W., Zurawski, G., and de Vries, J. E. Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes: Comparison with IL-4 and modulation by IFN-gamma or IL-10. *J. Immunol.* 151:6370-6381, 1993.

Debs, R. J., Fuchs, H. J., Philip, R., Montgomery, A. B., Brunette, E. N., Liggitt, D., Patton, J., and Shellito, J. E. Lung-specific delivery of cytokines induces sustained pulmonary and systemic immunomodulation in rats. *J. Immunol.* 140:3482-3488, 1988.

Del Prete, G., de Carli, M., Almerigogna, F., Giudizi, M. G., Biagiotti, R., and Romagnani, S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J. Immunol.* 150:353-360, 1993.

Ding, L., Linsley, D. S., Huang, L., Germain, R. N., and Shervach, E. M. IL-10 inhibits macrophage co-stimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J. Immunol.* 151:1224-1234, 1993.

Ding, L. and Shevach, E. M. IL-10 inhibits mitogen induced T cell proliferation by selectively inhibiting macrophage costimulatory function. *J. Immunol.* 148(10):3133-3139, 1992.

Doherty, G. M., Lange, J. R., Langstein, H. N., Alexander, H. R., Buresh, C. M., and Norton, J. A. Evidence for IFN-gamma as a mediator of the lethality of endotoxin and tumor necrosis factor- α . *J. Immunol.* 149(5):1666-1670, 1992.

Donnelly, S. C., Strieter, R. M., Kunkel, S. L., Walz, A., Robertson, C. R., Carter, D. C., Grant, I. S., Pollock, A. J., and Haslett, C. Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. *Lancet* 341(8846):643-647, 1993.

Donnelly, S. C., Strieter, R. M., Kunkel, S. L., Burdick, M., Armstrong, I., Kelly, K., Reid, P. T., and Haslett, C. Prognostic implications of interleukin-10 (IL-10) in bronchoalveolar lavage (BAL) samples in the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 151:A75 1995.(Abstract)

Downey, G. P., Worthen, G. S., Henson, P. M., and Hyde, D. M. Neutrophil sequestration and migration in localised pulmonary inflammation. *Am. Rev. Respir. Dis.* 147:168-176, 1993.

du Bois, R. M. Fibrosing alveolitis. *Practitioner* 237:782-788, 1993.

Egan, J. J., Stewart, J. P., Hasleton, P. S., Arrand, J. R., Carroll, K. B., and Woodcock, A. A. Epstein Barr virus replication within type II alveolar cells in idiopathic pulmonary fibrosis. *Am. Rev. Respir. Dis.* 151:a691 1995.(Abstract)

Eichaker, P. Q., Hoffman, W. D., Farese, A., Banks, S. M., Kuo, G. C., MacVittie, T. J., and Natanson, C. TNF but not IL-1 in dogs causes lethal lung injury and multiple organ dysfunction similar to human sepsis. *J. Appl. Physiol.* 71(5):1979-1989, 1991.

Elias, J. Tumor necrosis factor interacts with interleukin-1 and interferon to inhibit fibroblast proliferation via fibroblast prostaglandin-dependent and -independent mechanisms. *Am. Rev. Respir. Dis.* 138:652-658, 1988.

Elias, J. A., Schreiber, A. D., Gustilo, K., Chien, P., Rossman, M. D., Lammie, P. J., and Daniele, R. P. Differential interleukin 1 elaboration by unfractionated and density fractionated human alveolar macrophages and blood monocytes: Relationship to cell maturity. *J. Immunol.* 135(5):3198-3204, 1985.

Elias, J. A., Gustilo, K., and Freundlich, B. Human alveolar macrophage and blood monocyte inhibition of fibroblast proliferation - evidence for synergy between interleukin-1 and tumor necrosis factor. *Am. Rev. Respir. Dis.* 138:1595-1603, 1988.

Elliott, C. G., Rasmusson, B. Y., Crapo, R. O., Morris, A. H., and Jensen, R. L. Prediction of pulmonary function abnormalities after adult respiratory distress syndrome (ARDS). *Am. Rev. Respir. Dis.* 135:34-38, 1987.

Engelmann, H., Novick, D., and Wallach, D. Two tumor necrosis factor-binding proteins purified from human urine. *J. Biol. Chem.* 265(3):1531-1536, 1990.

Enk, A. H., Angeloni, V. L., Udey, M. C., and Katz, S. I. Inhibition of Langerhans cell antigen-presenting function by IL-10: A role for IL-10 in induction of tolerance. *J. Immunol.* 151:2390-2398, 1993.

Essner, R., Rhoades, K., McBride, W. H., Morton, D. L., and Economou, J. S. IL-4 downregulates IL-1 and TNF gene expression in human monocytes. *J. Immunol.* 142:3557-3561, 1989.

Everson, M. P. and Chandler, D. B. Changes in distribution, morphology, and tumor necrosis factor- α secretion of alveolar macrophage subpopulations during the development of bleomycin induced pulmonary fibrosis. *Am. J. Pathol.* 140:503-512, 1992.

Ferro, T. J., Kern, J. A., Elias, J. A., Kamoun, M., Daniele, R. P., and Rossman, M. D. Alveolar macrophages, blood monocytes and density-fractionated alveolar macrophages differ in their ability to promote lymphocyte proliferation to mitogen and antigen. *Am. Rev. Respir. Dis.* 135:682-687, 1987.

Fiers, W. Tumor necrosis factor: Characterisation at the molecular, cellular and in vivo level. *FEBS Letts.* 285(2):199-212, 1991.

Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M., and O'Garra, A. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* 147:3815-3822, 1991.

Florquin, S., Amraoui, Z., Abramowicz, D., and Goldman, M. Systemic release and protective role of IL-10 in staphylococcal enterotoxin B-induced shock in mice. *J. Immunol.* 153:2618-2623, 1994.

Foley, N., Lambert, C., McNicol, M., Johnson, N., and Rook, G. A. W. An inhibitor of the toxicity of tumour necrosis factor in the serum of patients with sarcoidosis, tuberculosis and Crohn's disease. *Clin. Exp. Immunol.* 80:395-399, 1990.

Foley, N. M., Millar, A. B., Meager, A., Johnson, N. M., and Rook, G. A. W. Tumour necrosis factor production by alveolar macrophages in pulmonary sarcoidosis and tuberculosis. *Sarcoidosis* 9:29-34, 1992.

Folkard, S. G., Westwick, J., and Millar, A. B. RANTES production by pulmonary macrophages distinguishes intrinsic from extrinsic asthma. *Clin. Exp. Allergy* (submitted):1995.

Fothergill, J., Bennet, D., Tighe, D., and Moss, R. The adult respiratory distress syndrome in septicemia. *Critical Care* June:465-474, 1987.

Fowler, A. A., Hamman, R. F., Good, J. T., Benson, K. N., Baird, M., Eberle, D. J., Petty, T. L., and Hyers, T. M. Adult respiratory distress syndrome: risk with common predispositions. *Ann. Internal Med.* 98:593-597, 1983.

Fowler, A. A., Hyers, T. M., Fisher, B. J., Bechard, D. E., Centor, R. M., and Webster, R. O. The adult respiratory distress syndrome: cell populations and soluble mediators in the air spaces of patients at high risk. *Am. Rev. Respir. Dis.* 136:1225-1231, 1987.

Frei, K., Nadal, D., Pfister, H.-W., and Fontana, A. Listeria meningitis: Identification of a cerebrospinal fluid inhibitor of macrophage listericidal function as interleukin 10. *J. Exp. Med.* 178:1255-1261, 1993.

Froon, A. H., Bemelmans, M. H., Greve, J. W., van der Linden, C. J., and Buurman, W. A. Increased plasma concentrations of soluble tumor necrosis factor receptors in sepsis syndrome. *Critical care medicine* 22:803-809, 1994.

Fry, J., White, R., and Whitfield, M. Respiratory Disorders. London: Churchill Livingstone, 1984.

Fuchs, H. J., Snizek, M., and Shellito, J. E. Cellular influx and activation increase macrophage cytotoxicity and interleukin-1 elaboration during pulmonary inflammation in rats. *Am. Rev. Respir. Dis.* 138:572-577, 1988.

Galve-de Rochemonteix, B., Nicod, L. P., Chicheportiche, R., Lacraz, S., Baumberger, C., and Dayer, J. Regulation of interleukin-1ra, interleukin-1 α , and interleukin-1 β production by human alveolar macrophages with phorbol myristate acetate, lipopolysaccharide, and interleukin-4. *Am. J. Respir. Cell Mol. Biol.* 8:160-168, 1993.

Gant, V. A., Shakoor, Z., Barbosa, I. L., and Hamblin, A. S. Normal and sarcoid alveolar macrophages differ in their ability to present antigen and to cluster with autologous lymphocytes. *Clin. Exp. Immunol.* 86:494-499, 1991.

Gerard, C., Bruyns, C., Marchant, A., Abramowicz, D., Vandenabeele, P., Delvaux, A., Fiers, W., Goldman, M., and Velu, T. Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxaemia. *J. Exp. Med.* 177:547-550, 1993.

Gessani, S., Testa, U., Varano, B., Di Marzio, P., Borghi, P., Conti, L., Barberi, T., Tritarelli, E., Martucci, R., Seripa, D., Peschle, C., and Belardelli, F. Enhanced production of LPS-induced cytokines during differentiation of human monocytes to macrophages. *J. Immunol.* 151:3758-3766, 1993.

Han, J., Brown, T., and Beutler, B. Endotoxin-responsive sequences control cachectin tumour necrosis. Biosynthesis at the translational level. *J. Exp. Med.* 171:1990.

Han, J., Beutler, B., and Huez, G. Complex regulation of tumor necrosis factor mRNA turnover in lipopolysaccharide-activated macrophages. *Biochim. Biophys. Acta* 1090:22-28, 1991a.

Han, J., Huez, G., and Beutler, B. Interactive effects of the tumor necrosis factor promoter and 3' untranslated regions. *J. Immunol.* 146:1843-1850, 1991b.

Hance, A. H., Douches, S., Winchester, R. J., Ferrans, V. J., and Crystal, R. G. Characterisation of mononuclear phagocyte subpopulations in the human lung by using monoclonal antibodies: changes in alveolar macrophage phenotype associated with pulmonary sarcoidosis. *J. Immunol.* 134(1):284-292, 1985.

Haran, N., Bar-Khayim, Y., Frensdorff, A., and Barnard, G. Tumor necrosis factor binding protein: interference in immunoassays of TNF. *Kidney Int.* 40:1166-1170, 1991.

Hällgren, R., Samuelsson, T., Venge, P., and Modig, J. Eosinophil activation in the lung is related to lung damage in adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 135:639-642, 1987.

Heilig, B., Wermann, M., Gallati, H., Brockhaus, M., Berke, B., Egen, O., and Pezzutto, A. Elevated TNF receptor plasma concentrations in patients with rheumatoid arthritis. *Clin. Investig.* 70:22-27, 1992.

Heilig, B., Pezzutto, A., Lukoschek, M., and Hunstein, W. Expression of TNF receptors in rheumatoid arthritis and ankylosing spondylitis. *z. rheumatol.* 52:383-389, 1993.

Hempel, S. L., Monick, M. M., and Hunninghake, G. W. Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. *J. Clin. Invest.* 93:391-396, 1994.

Henke, C., Marineili, W., Jessurun, J., Fox, J., Harms, D., Peterson, M., Chiang, L., and Doran, P. Macrophage production of basic fibroblast growth factor in the fibroproliferative disorder of alveolar fibrosis after lung injury. *Am. J. Pathol.* 143:1189-1199, 1993.

Henson, P. M. and Riches, D. W. H. Modulation of macrophage maturation by cytokines and lipid mediators: a potential role in resolution of pulmonary inflammation. *Ann. NY Acad. Sci.* 298-308, 1994.

Herbert, J. M., Savi, P., Laplace, M. C., Lale, A., Dol, F., Dumas, A., Labit, C., and Minty, A. IL-4 and IL-13 exhibit comparable abilities to reduce pyrogen-induced expression of procoagulant activity in endothelial cells and monocytes. *FEBS Letts.* 328:268-270, 1993.

Hert, R. and Albert, R. K. Sequelae of the adult respiratory distress syndrome. *Thorax* 49:8-13, 1994.

Holt, P. G., Oliver, J., Bilyk, N., McMenamin, P. G., Kraal, G., and Thepen, T. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J. Exp. Med.* 177:397-407, 1993.

Horvath, C. J., Ferro, T. J., Jesmok, G., and Malik, A. B. Recombinant tumor necrosis factor increases pulmonary vascular permeability independent of neutrophils. *Proc. Natl. Acad. Sci. USA* 85:9219-9223, 1988.

Howard, M., Muchamuel, T., Andrade, S., and Menon, S. Interleukin 10 protects mice from lethal endotoxaemia. *J. Exp. Med.* 177:1205-1208, 1993.

Howard, M. and O'Garra, A. Biological properties of interleukin 10. *Immunol. Today* 13:198-200, 1992.

Hudspith, B. N., Flint, K. C., Geraint-James, D., Brostoff, J., and McI Johnson, N. Lack of immune deficiency in sarcoidosis: compartmentalisation of the immune response. *Thorax* 42:250-255, 1987.

Hunninghake, G. W. Release of interleukin-1 by alveolar macrophages of patients with active pulmonary sarcoidosis. *Am. Rev. Respir. Dis.* 129:569-572, 1984.

Hunninghake, G. W. and Crystal, R. G. Pulmonary sarcoidosis. *N. Engl. J. Med.* 305:429-434, 1981.

Hyers, T. M., Gee, M., and Andreadis, N. A. Cellular interactions in the multiple organ injury syndrome. *Am. Rev. Respir. Dis.* 135:952-953, 1987.

Ihle, J.N. STATs: Signal transducers and activators of transcription. *Cell* 84:331-334, 1996.

Ina, Y., Takada, K., Yamamoto, M., Morishita, M., and Miyachi, A. Antigen-presenting capacity of patients with sarcoidosis. *Chest* 98:911-916, 1990.

Ishida, H., Muchamuel, T., Sakaguchi, S., Andrade, S., Menon, S., and Howard, M. Continuous administration of anti-interleukin 10 antibodies delays onset of autoimmunity in NZB/W F₁ mice. *J. Exp. Med.* 179:305-310, 1994.

Ito, C. Y., Kazantsev, A. G., and Baldwin, A. S., Jr. Three NF-kappa B sites in the Ikappa B-alpha promoter are required for induction of gene expression by TNF α . *Nucleic Acids Res.* 22:3787-3792, 1994.

Itoh, A., Yamaguchi, E., Kuzumaki, N., Okazaki, N., Furuya, K., Abe, S., and Kawakami, Y. Expression of Granulocyte-macrophage Colony-stimulating factor mRNA by inflammatory cells in the sarcoid lung. *Am. J. Respir. Cell Mol. Biol.* 3:245-249, 1990.

Itoh, A., Yamaguchi, E., Furuya, K., Hizawa, N., Ohnuma, N., Kawakami, Y., and Kuzumaki, N. Correlation of GM-CSF mRNA in bronchoalveolar fluid with indices of clinical activity in sarcoidosis. *Thorax* 48:1230-1234, 1993.

Jablons, D. M., Mulé, J. J., McIntosh, J. K., Sehgal, P. B., May, L. T., Huang, C. M., Rosenberg, S. A., and Lotz, M. T. IL-6/IFN- β -2 as a circulating hormone. *J. Immunol.* 142:1542-1547, 1989.

Jacobs, R. F., Tabor, D. R., Burks, A. W., and Campbell, G. D. Elevated interleukin-1 release by human alveolar macrophages during the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 140:1686-1692, 1989.

Janossy, G., Bofill, M., Poulter, L.W., Rawlings, E., Burford, G.D., Navarette, C., Ziegler, A., and Keleman, E. Separate ontogeny of two macrophage-like accessory cell populations in the human foetus. *J. Immunol.* 136:4354-4361, 1986.

Jonas, E., Sargent, T. D., and Davis, I. B. Epidermal keratin-gene expressed in embryos of xenopus laevis. *Proc. Natl. Acad. Sci. USA* 82:5413 1985.

Joyce, D. A., Gibbons, D. P., Green, P., Steer, J. H., Feldmann, M., and Brennan, F. M. Two inhibitors of pro-inflammatory cytokine release, IL-10 and IL-4, have contrasting

effects on release of soluble p75 tumor necrosis factor receptor by cultured monocytes. *Eur. J. Immunol.* 24:2699-2705, 1995.

Kalthoff, H., Roeder, C., Brockhaus, M., Thiele, H., and Schmiegel, W. Tumor necrosis factor (TNF) up-regulates the expression of p75 but not p55 TNF receptors, and both receptors mediate, independently of each other, up-regulation of transforming growth factor α and epidermal growth factor receptor mRNA. *J. Biol. Chem.* 268:2762-2766, 1993.

Kasama, T., Strieter, R. M., Lukacs, N. W., Burdick, M. D., and Kunkel, S. L. Regulation of neutrophil-derived chemokine expression by IL-10. *J. Immunol.* 152:3559-3569, 1994.

Katsikis, P. D., Chu, C.-Q., Brennan, F. M., Maini, R. N., and Feldmann, M. Immunoregulatory role of interleukin 10 in rheumatoid arthritis. *J. Exp. Med.* 179:1517-1527, 1994.

Kawanami, O., Ferrans, V. J., and Fulmer, J. D. Nuclear inclusions in alveolar epithelium of patients with fibrotic lung disorders. *Am. J. Pathol.* 94:301-312, 1979.

Keele, C. A., Neil, E., and Joels, N. Samson Wright's Applied Physiology. Oxford: Oxford University Press, 1982.

Kelley, J. Cytokines of the lung. *Am. Rev. Respir. Dis.* 141:765-788, 1990.

Kelso, A. Th1 and Th2 subsets: paradigms lost? *Immunol. Today* 16:374-379, 1995.

Klebanoff, S. J., Vadas, M. A., Harlan, J. M., Sparks, L. H., Gamble, J. R., Agosti, J. M., and Waltersdorff, A. M. Stimulation of neutrophils by tumor necrosis factor. *J. Immunol.* 136:4220-4225, 1986.

Kline, J. N., Schwartz, D. A., Monick, M. M., Floerchinger, C. S., and Hunninghake, G. W. Relative release of interleukin-1 β and interleukin-1 receptor antagonist by alveolar macrophages. *Chest* 104:47-53, 1993.

Kohase, M., Henriksen-Destafano, D., Sehgal, P. B., and Vilcek, J. Dexamethasone inhibits feedback regulation of the mitogenic activity of tumor necrosis factor, interleukin-1, and epidermal growth factor in human fibroblasts. *J. Cell Physiol.* 132:271-278, 1987.

Kotloff, R. M., Little, J., and Elias, J. A. Human alveolar macrophage and blood monocyte interleukin-6 production. *Am. J. Respir. Cell Mol. Biol.* 3:497-505, 1990.

Krakauer, T. and Oppenheim, J. J. IL-1 and tumor necrosis factor- α each up-regulate both the expression of IFN-gamma receptors and enhance IFN-gamma-induced HLA-DR expression on human monocytes and a human monocytic cell line (THP-1). *J. Immunol.* 150:1205-1211, 1993.

Krönke, M., Schütze, S., Scheurich, P., Meichle, A., Hensel, G., Thoma, B., Kruppa, G., and Pfizenmaier, K. Tumour necrosis factor signal transduction. *Cell Signalling* 2:1-8, 1990.

Kuhns, D. B., Alvord, W. G., and Gallin, J. I. Increased circulating cytokines, cytokine antagonists, and E-selectin after intravenous administration of endotoxin in humans. *J. Infect. Dis.* 171:145-152, 1995.

Larrick, J. W., Fischer, D. G., Anderson, J., and Koren, H. S. Characterization of a human macrophage-like cell line stimulated in vitro: a model of macrophage function. *J. Immunol.* 125:6-12, 1980.

Lee, C. T., Fein, A. M., Lippmann, M., Holtzman, H., Kimbel, P., and Weinbaum, G. Elastolytic activity in pulmonary lavage fluid from patients with adult respiratory distress syndrome. *N. Engl. J. Med.* 304(4):192-195, 1981.

Leeuwenberg, J. F., Dentener, M. A., and Buurman, W. A. Lipopolysaccharide LPS-mediated soluble TNF receptor release and TNF receptor expression by monocytes. *J. Immunol.* 152:5070-5076, 1994.

Leff, J. A., Parsons, P. E., Day, C. E., Taniguchi, N., Jochum, M., Fritz, H., Moore, F. A., Moore, E. E., McCord, J. M., and Repine, J. E. Serum antioxidants as predictors of adult respiratory distress syndrome in patients with sepsis. *Lancet* 341:777-780, 1993.

Lem, V. M., Lipscomb, M. F., Weissler, J. C., Nunez, G., Ball, E. J., Stastny, P., and Toews, G. B. Bronchoalveolar cells from sarcoid patients demonstrate enhanced antigen presentation. *J. Immunol.* 135(3):1766-1771, 1985.

Liabakk, N., Sundan, A., Lien, E., Laegreid, A., Bombara, M. P., Ranges, G., and Espevik, T. The release of soluble p55 TNF receptor from U937 cells studied by a new p55 immunoassay. *J. Immunol. Methods* 163:145-154, 1993.

Libby, D. M., Gibofsky, A., and Fotino, M. Immunogenetic and clinical findings in IPF association with the B-cell alloantigen HLA-DR2. *Am. Rev. Respir. Dis.* 141:307-313, 1983.

Lin, H. S., Lokeshwar, B. I., and Hsu, S. Both granulocyte macrophage CSF and monocyte CSF control the proliferation and survival of the same subset of alveolar macrophages. *J. Immunol.* 142:515-519, 1989.

Lo, S. K., Everitt, J., Gu, J., and Malik, A. B. Tumor necrosis factor mediates experimental pulmonary edema by ICAM-1 and CD18-dependent mechanisms. *J. Clin. Invest.* 89:981-988, 1992.

Macatonia, S. E., Doherty, T. M., Knight, S. C., and O'Garra, A. Differential effect of IL-10 on dendritic cell-induced T cell proliferation and IFN-gamma production. *J. Immunol.* 150:3755-3765, 1993.

MacNee, W. and Selby, C. Neutrophil traffic in the lungs: role of haemodynamics, cell adhesion, and deformability. *Thorax* 48:79-88, 1993.

Mantovani, A. and Dejana, E. Cytokines as communication signals between leukocytes and endothelial cells. *Immunol. Today* 10:370-375, 1989.

Marchant, A., Deviere, J., Byl, B., de Groote, D., Vincent, J., and Goldman, M. IL-10 production during septicemia. *Lancet* 343:707-708, 1994.

Marianayagam, L. and Poulter, L. W. Corticosteroid can alter antigen expression on alveolar macrophages. *Clin. Exp. Immunol.* 85:531-535, 1991.

Marks, J. D., Marks, C. B., Luce, J. M., Montgomery, A. B., Turner, J., Metz, C. A., and Murray, J. F. Plasma tumour necrosis factor in patients with septic shock. *Am. Rev. Respir. Dis.* 141:94-97, 1990.

Martinet, Y., Yamauchi, K., and Crystal, R. G. Differential expression of the tumour necrosis factor/cachectin gene by blood and lung mononuclear phagocytes. *Am. Rev. Respir. Dis.* 139:659-665, 1988.

Matsushima, K. and Oppenheim, J. J. Interleukin 8 and MCAF: Novel Inflammatory cytokines inducible by IL-1 and TNF. *Cytokine* 1:2-13, 1989.

Mayernik, D. G., Ul-Haq, A., and Rinehart, J. J. Differentiation-associated alteration in human monocyte-macrophage accessory cell function. *J. Immunol.* 130(5):2156-2160, 1983.

McKenzie, A. N. J., Culpepper, J. A., de Waal Malefyt, R., Briere, F., Punnonen, J., Aversa, G., Sato, A., Dang, W., Cocks, B. G., Menon, S., de Vries, J. E., Banchereau, J., and Zurawski, G. Interleukin-13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proc. Natl. Acad. Sci. USA* 90:3735-3739, 1993.

Michie, H. R., Manogue, K. R., Spriggs, D. R., Revhaug, A., O'Dwyer, S., Dinarello, C. A., Cerami, A., Wolff, S. M., and Wilmore, D. W. Detection of circulating tumor necrosis factor after endotoxin administration. *N. Engl. J. Med.* 318:1481-1486, 1988.

Millar, A. B., Singer, M., Meager, A., Foley, N. M., Johnson, N. M., and Rook, G. A. W. Tumor necrosis factor in bronchopulmonary secretions of patients with adult respiratory-distress syndrome. *Lancet* 2:712-714, 1989.

Miller, E. J., Cohen, A. B., Nagao, S., Griffith, D., Maunder, R. J., Martin, T. R., Weiner-Kronish, J. P., Sticherling, M., Christophers, E., and Matthay, M. A. Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. *Am. Rev. Respir. Dis.* 146:427-432, 1992.

Miller, K. S. Lung disease associated with progressive systemic sclerosis. *Am. Rev. Respir. Dis.* 141:301-306, 1990.

Mohler, K. M., Torrance, D. S., Smith, C. A., Goodwin, R. G., Stremter, K. E., Fung, V. P., Madari, H., and Widmer, M. B. Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxaemia and function simultaneously as both TNF carriers and TNF antagonists. *J. Immunol.* 151:1548-1561, 1993.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. Two types of murine helper T cell clone. *J. Immunol.* 136:2348-2359, 1986.

Muldoon, S. R., Hinton, K., and Calhoun, W. J. Modulation of IL-1 β and TNF- α by IL-10 in peripheral blood monocytes - evidence of down-regulation. *Am. J. Respir. Crit. Care Med.* 149:A1099, 1994.

Muller-Quernheim, J., Pfeifer, S., Mannel, D., Strausz, J., and Ferlinz, R. Lung-restricted activation of the alveolar macrophage/monocyte system in pulmonary sarcoidosis. *Am. Rev. Respir. Dis.* 145:187-192, 1992.

Mulligan, M. S., Jones, M. L., Vaporciyan, A. A., Howard, M. C., and Ward, P. A. Protective effects of IL-4 and IL-10 against immune complex-induced lung injury. *J. Immunol.* 151:5666-5674, 1993.

Munro, C. S. and Mitchell, D. N. The Kveim response: still useful, still a puzzle. *Thorax* 42:321-331, 1987.

Murray, J. F., Matthay, M. A., Luce, J. M., and Flick, M. R. An expanded definition of the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 138:720-723, 1988.

Nakane, A., Minagawa, T., Kahanawa, M., Chen, Y., Sato, H., Manyama, M., and Tsuruoka, N. Interactions between endogenous gamma-interferon and tumor necrosis factor-alpha in host resistance against primary and secondary *Listeria monocytogenes* infection. *Infect. Immun.* 57:3331-3337, 1989.

Nakata, K., Akagawa, K. S., Fukayama, M., Hayashi, Y., Kadokura, M., and Tokunaga, T. Granulocyte-Macrophage Colony -Stimulating Factor promotes the proliferation of human alveolar macrophages in vitro. *J. Immunol.* 147:1266-1272, 1991.

Nathan, C. F. Secretory products of macrophages. *J. Clin. Invest.* 79:319-326, 1987.

Nathan, C. F. Respiratory burst in adherent human neutrophils: triggering by colony-stimulating factors CSF-GM and CSF-G. *Blood* 73:301-306, 1989.

Nawroth, P. P. and Stern, D. M. Modulation of endothelial cell haemostatic properties by tumour necrosis factor. *J. Exp. Med.* 163:740-745, 1986.

Nishioka, Y., Sone, S., Orino, E., Nii, A., and Ogura, T. Down-regulation by interleukin 4 of activation of human alveolar macrophages in the tumoricidal state. *Cancer Res.* 51:5526-5531, 1991.

Noble, B., du Bois, R. M., and Poulter, L. W. The distribution of phenotypically distinct macrophage subsets in the lungs of patients with cryptogenic fibrosing alveolitis. *Clin. Exp. Immunol.* 76:41-46, 1989.

Obrig, T. G., Gulp, W. J., McKeehan, W. L., and Hordesty, B. The mechanisms by which cyclohexamide and related glutamide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. *J. Biol. Chem.* 246:174-178, 1971.

Ogawa, H., Kunkel, S. L., Fantone, J. C., and Ward, P. A. Comparative study of eosinophil and neutrophil chemotaxis and enzyme release. *Am. J. Pathol.* 105:149-155, 1981.

Oswald, I. P., Gazzinelli, R. T., Sher, A., and James, S. L. IL-10 synergizes with IL-4 and transforming growth factor beta to inhibit macrophage cytotoxic activity. *J. Immunol.* 148(11):3578-3582, 1992a.

Oswald, I. P., Wynn, T. A., Sher, A., and James, S. L. Interleukin 10 inhibits macrophage microbicidal activity by blocking endogenous production of tumor necrosis factor α required as a costimulatory factor for interferon gamma-induced activation. *Proc. Natl. Acad. Sci. USA* 89:8676-8680, 1992b.

Paleolog, E. M., De La Salle, S. A., Buurman, W. A., and Feldmann, M. Functional activities of receptors for tumor necrosis factor-alpha on human vascular endothelial cells. *Blood* 84:2578-2590, 1994.

Parson, P. E., Moore, F. A., Moore, E. E., Iklé, D. N., Henson, P. M., and Worthen, G. S. Studies on the role of tumor necrosis factor in adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 146:694-700, 1992.

Paul, W. E. Interleukin 4/B cell stimulatory factor 1: one lymphokine, many functions. *FASEB J.* 1:456-461, 1987.

Pepe, P. E., Potkin, R. T., Holtman Reus, D., Hudson, L. D., and Carrico, C. J. Clinical predictors of the adult respiratory distress syndrome. *Am. J. Surgery* 144:124-128, 1982.

Piguet, P. F., Collart, M. A., Grau, G. E., Kapanci, Y., and Vassalli, P. Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. *J. Exp. Med.* 170:655-663, 1989.

Piguet, P. F., Collart, M. A., Grau, G. E., Sappino, A., and Vassalli, P. Requirement of tumour necrosis factor for development of silica-induced pulmonary fibrosis. *Nature* 344:245-247, 1990.

Piguet, P. F., Miyazaki, Y., Araki, K., Vesin, C., Garcia, I., Kapanci, Y., Whitsett, J. A., and Vassalli, P. Pulmonary fibrosis in transgenic mice over-expressing TNF- α in alveolar type II pneumocytes. *Am. Rev. Respir. Dis.* 151:A8291995.(Abstract)

Popp, W. and Wachtler, F. J. Correlation of morphological patterns of nucleoli in alveolar macrophages with HLA-DR antigen expression in sarcoidosis. *Thorax* 46:878-880, 1991.

Porteau, F. and Hieblot, C. Tumor necrosis factor induces a selective shedding of its p75 receptor from human neutrophils. *J. Biol. Chem.* 269:2834-2840, 1994.

Porteu, F. and Nathan, C. Shedding of tumor necrosis factor receptors by activated human neutrophils. *J. Exp. Med.* 172:599-607, 1990.

Poulter, L. W. Antigen presenting cells *in situ*: their identification and involvement in immunopathology. *Clin. Exp. Immunol.* 53:513-520, 1983.

- Poulter, L. W. Changes in lung macrophages during disease. *FEMS* 64:327-332, 1990.
- Powrie, F., Menon, S., and Coffman, R. L. Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity *in vivo*. *Eur. J. Immunol.* 23:3043-3049, 1993.
- Pratt, V. C., Vollmer, R. T., Shelburne, J. D., and Crapo, J. D. Pulmonary morphology in a multi-hospital collaborative extra-corporeal membrane oxygenation project. *Am. J. Pathol.* 95:191-214, 1979.
- Rabinovici, R., Feuerstein, G., and Neville, L. F. Cytokine gene and peptide regulation in lung microvascular injury: new insights on the development of adult respiratory distress syndrome. *Ann. NY Acad. Sci.* 346-353, 1994.
- Ralph, P., Nakoinz, I., Sampson-Johannes, A., Fong, S., Lowe, D., Min, H., and Lin, L. IL-10, T lymphocyte inhibitor of human blood cell production of IL-1 and Tumor necrosis factor. *J. Immunol.* 148(3):808-814, 1992.
- Reitamo, S., Remitz, A., Tamai, K., Ledo, I., and Uitto, J. Interleukin 10 up-regulates elastin gene expression *in vivo* and *in vitro* at the transcriptional level. *Biochem. J.* 302:331-333, 1994.
- Repine, J. E. Scientific perspectives on adult respiratory distress syndrome. *Lancet* 339:466-469, 1992.
- Rinaldo, J. E. and Rogers, R. M. Adult respiratory distress syndrome. Changing concepts of lung injury and repair. *N. Engl. J. Med.* 306:900-909, 1982.
- Robinson, B. W. S., McLemore, T. L., and Crystal, R. G. Gamma interferon is spontaneously released by alveolar macrophages and lung T lymphocytes in patients with pulmonary sarcoidosis. *J. Clin. Invest.* 75:1488-1495, 1985.

Rocker, G. M., Pearson, D., Wiseman, M. S., and Shale, D. J. Diagnostic criteria for adult respiratory distress syndrome: time for reappraisal. *Lancet* Jan 21:120-123, 1989.

Roitt, I., Brostoff, J., and Male, D. Immunology. London: Churchill Livingstone, 1989.

Rolfe, M. W., Kunkel, S. L., Standiford, T. J., Chensue, S. W., Allen, R. M., Evanoff, H. L., Phan, S. H., and Strieter, R. M. Pulmonary fibroblast expression of interleukin-8: A model for alveolar macrophage-derived cytokine networking. *Am. J. Respir. Cell Mol. Biol.* 5:493-501, 1991.

Rolfe, M. W., Standiford, T. J., Kunkel, S. L., Burdick, M. D., Gilbert, A. R., Lynch, J. P., and Strieter, R. M. Interleukin-1 receptor antagonist expression in sarcoidosis. *Am. Rev. Respir. Dis.* 148:1378-1384, 1993.

Rook, G. A. W., Taverne, J., Leveton, C., and Steele, J. The role of gamma-interferon, vitamin D₃ metabolites and tumour necrosis factor in the pathogenesis of tuberculosis. *Immunology* 62:229-234, 1987.

Rose, C. E., Juliano, C. A., Tracey, D. E., Yoshimura, T., and Fu, S. M. Role of interleukin-1 in endotoxin-induced lung injury in the rat. *Am. J. Respir. Cell Mol. Biol.* 10:214-221, 1994.

Rossaint, R., Falke, K. J., Lopez, F., Slama, K., Pison, U., and Zapol, W. M. Inhaled nitric oxide for the adult respiratory distress syndrome. *N. Engl. J. Med.* 328(6):399-405, 1993.

Roten, R., Markert, M., Feihl, F., Schaller, M., Tagan, M., and Perret, C. Plasma levels of tumor necrosis factor in the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 143:590-592, 1991.

Sandoz, E. Uber zwei faller von fotaler bronchektasie. *Beitr. Pathol. Anat* 1907.

Schaberg, T., Rau, M., Stephan, H., and Lode, H. Increased number of alveolar macrophages expressing surface molecules of the CD11/CD18 family in sarcoidosis and idiopathic pulmonary fibrosis is related to the production of superoxide anions by these cells. *Am. Rev. Respir. Dis.* 147:1507-1513, 1993.

Schall, T. J., Bacon, K., Camp, R. D. R., Kaspari, J. W., and Goeddel, D. V. Human macrophage inflammatory protein alpha (MIP-1 α) and MIP-1 β chemokines attract distinct populations of lymphocytes. *J. Exp. Med.* 177:1821-1825, 1993.

Schollmeier, K. Immunologic and pathophysiologic role of tumor necrosis factor. *Am. J. Respir. Cell Mol. Biol.* 3:11-12, 1990.

Schumann, R. R., van der Bosch, J., Rüller, S., Ernst, S., and Schlaak, M. Monocyte long term cultivation on microvascular endothelial cell monolayers: Morphologic and phenotypic characterization and comparison with monocytes cultured on tissue culture plastic. *Blood* 3:818-826, 1989.

Semenzato, G. The immunology of sarcoidosis. *Semin. Respir. Med.* 8:17-29, 1986.

Serra, M. C., Calzetti, F., Ceska, M., and Cassatella, M. A. Effect of substance P on superoxide anion and IL-8 production by human PMNL. *Immunology* 82:63-69, 1994.

Sewell, K. L. and Trentham, D. E. Pathogenesis of rheumatoid arthritis. *Lancet* 341:283-286, 1993.

Shapiro, L., Clark, B. D., Orencole, S. F., Poutsika, D. D., Granowitz, E. V., and Dinarello, C. A. Detection of tumor necrosis factor soluble receptor p55 in blood samples from healthy and endotoxaemic humans. *J. Infect. Dis.* 167:1344-1350, 1993.

Sharma, O. P. and Johnson, R. Airway obstruction in a study of 123 non-smoking black American patients with sarcoidosis. *Chest* 94:343-346, 1988.

Shaw, G. and Kamen, R. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* 46:659-667, 1986.

Sieling, P. A., Abrams, J. S., Yamamura, M., Salgame, P., Bloom, B. R., Rea, T. H., and Modlin, R. L. Immunosuppressive roles for IL-10 and IL-4 in human infection. In vitro modulation of T cell responses in leprosy. *J. Immunol.* 150:5501-5510, 1993.

Smith, S. R., Terminelli, C., Kenworthy-Bott, L., Calzetta, A., and Donkin, J. The cooperative effects of TNF- α and IFN- γ are determining factors in the ability of IL-10 to protect mice from lethal endotoxemia. *J. Leukocyte Biol.* 55:711-718, 1994.

Sobell, H. M. Actinomycin D and DNA transcription. *Proc. Natl. Acad. Sci. USA* 82:5328-5331, 1985.

Spatafora, M., Merendino, A., Chiappara, G., Gjomarkaj, M., Melis, M., Bellia, V., and Bonsignore, G. Lung compartmentalisation of increased TNF releasing ability by mononuclear phagocytes in pulmonary sarcoidosis. *Chest* 96:542-549, 1989.

Spinas, G. A., Keller, U., and Brockhaus, M. Release of soluble receptors for tumor necrosis factor (TNF) - in relation to circulating TNF during experimental endotoxinaemia. *J. Clin. Invest.* 90:533-536, 1992.

Spiteri, M. and Poulter, L. W. Characterisation of immune inducer and suppresser macrophages from the normal human lung. *Clin. Exp. Immunol.* 83:157-162, 1991.

Spiteri, M. A., Clarke, S. W., and Poulter, L. W. Alveolar macrophages that suppress T-cell responses may be crucial to the pathogenetic outcome of pulmonary sarcoidosis. *Eur. Respir. J.* 5:394-403, 1992.

Spiteri, M. A., Clarke, S. W., and Poulter, L. W. Isolation of phenotypically and functionally distinct macrophage subpopulations from human bronchoalveolar lavage. *Eur. Respir. J.* 5:717-726, 1992.

Stack, B. H. R., Choo-Kang, Y. F. J., and Heard, B. E. The prognosis of cryptogenic fibrosing alveolitis. *Thorax* 27:535-542, 1972.

Standiford, T. J., Rolfe, M. W., Kunkel, S. L., Lynch, J. P., Burdick, M. D., Gilbert, A. R., Orringer, M. B., Whyte, R. I., and Strieter, R. M. Macrophage inflammatory protein-1 α expression in interstitial lung disease. *J. Immunol.* 151:2852-2863, 1993.

Steffen, M., Petersen, J., Oldigs, M., Karmeier, A., Magnussen, H., Thiele, H. G., and Raedler, A. Increased secretion of tumor necrosis factor-alpha, interleukin-1-beta, and interleukin-6 by alveolar macrophages from patients with sarcoidosis. *J. Allergy Clin. Immunol.* 91:939-949, 1993.

Stephens, K. E., Ishizaka, A., Larrick, J. W., and Raffin, T. A. Tumor necrosis factor causes increased pulmonary permeability and edema. *Am. Rev. Respir. Dis.* 137:1364-1370, 1988.

Stites, D. P. and Terr, A. I. Basic And Clinical Immunology. London: Prentice Hall, 1987.

Strieter, R. M., Remick, D. G., Lynch III, J. P., Raiford, G. M., Spengler, R., and Kunkel, S. L. Differential regulation of tumor necrosis factor-alpha in human alveolar macrophages and peripheral blood monocytes: a cellular and molecular analysis. *Am. J. Respir. Cell Mol. Biol.* 1:57-63, 1989a.

Strieter, R. M., Remick, D. G., Lynch, J. P., Spengler, R. N., and Kunkel, S. L. Interleukin-2-induced tumor necrosis factor-alpha gene expression in human alveolar macrophages and blood monocytes. *Am. Rev. Respir. Dis.* 139:335-342, 1989b.

Studdy, P. R. *Sarcoidosis*, in Respiratory Medicine. London. Balliere Tindall, 1990

Sugarman, B. J., Aggarwal, B. B., Hass, P. E., Figari, I. S., Palladino, M. A., and Shepard, H. M. Recombinant human tumor necrosis factor-alpha - effects on proliferation of normal and transformed cells in vitro. *Science* 230:943-945, 1985.

Suter, P. M., Suter, S., Girardin, E., Roux-Lombard, P., Grau, G. E., and Dayer, J. High bronchoalveolar levels of tumour necrosis factor and its inhibitors, interleukin-1, interferon, and elastase, in patients with adult respiratory distress syndrome after trauma, shock or sepsis. *Am. Rev. Respir. Dis.* 145:1016-1022, 1992.

Tartaglia, L. A., Goeddel, D. V., Reynolds, C., Figari, I. S., Weber, R. F., Fendly, B. M., and Palladino, M. A. Stimulation of human T-cell proliferation by specific activation of the 75 kDa tumor necrosis factor receptor. *J. Immunol.* 151:4637-4641, 1993.

Taylor, M. J., Hehnke, K., and Clark, C. L. Lipopolysaccharide and a phorbol ester stimulate secretion of tumor necrosis factor- α from alveolar macrophages through action on overlapping subsets of cells. *J. Leukocyte Biol.* 54:384-388, 1993.

Terao, I., Hashimoto, S., and Horie, T. Effect of GM-CSF on TNF-alpha and IL-1-beta production by alveolar macrophages and peripheral blood monocytes from patients with sarcoidosis. *Int. Arch. Allergy Immunol.* 102:242-248, 1993.

Thepen, T., Kraal, G., and Holt, P. G. The role of alveolar macrophages in regulation of lung inflammation. *Ann. NY Acad. Sci.* 200-206, 1994.

Thelen, M., Dewald, B., and Baggiolini, M. Neutrophil signal transduction and activation of the respiratory burst. *Physiol. Rev.* 73:797-821, 1993.

Thomas, P. D. and Hunninghake, G. W. Current concepts of the pathogenesis of sarcoidosis. *Am. Rev. Respir. Dis.* 135:747-760, 1987.

Thorne, K. J. I. and Mazza, G. Eosinophilia, activated eosinophils and human schistosomiasis. *J. Cell Sci.* 98:265-270, 1991.

Thornhill, M. H. and Haskard, D. O. IL-4 regulates endothelial cell activation by IL-1, tumor necrosis factor, or IFN-gamma. *J. Immunol.* 145:865-872, 1990.

Thrall, R. S., Barton, R. W., D'Amato, D. A., and Sulavik, S. B. Differential cellular analysis of bronchoalveolar lavage fluid obtained at various stages during the development of bleomycin-induced pulmonary fibrosis in the rat. *Am. Rev. Respir. Dis.* 126:488-492, 1982.

Toews, G. B. Pulmonary dendritic cells: Sentinels of lung-associated lymphoid tissues. *Am. J. Respir. Cell Mol. Biol.* 4:204-205, 1991.

Tomioka, M., Goto, T., Lee, T. D. G., Bienenstock, J., and Befus, A. D. Isolation and characterization of lung mast cells from rats with bleomycin-induced pulmonary fibrosis. *Immunology* 66:439-444, 1989.

Tracey, K. J., Beutler, B., Lowry, S. F., Merryweather, J., Wolpe, S., Milsark, I. W., Hariri, R. J., Fahey, T. J., Zentella, A., Albert, J., Shires, G. T., and Cerami, A. Shock and tissue injury induced by recombinant human cachectin. *Science* 234:470-474, 1986.

Tracey, K. J., Fong, Y., Hesse, D. G., Manogue, K. R., Lee, A. T., Kuo, G. C., Lowry, S. F., and Cerami, A. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature* 330:662-664, 1987a.

Tracey, K. J., Lowry, S. F., Fahey, T. J., Albert, J. D., Fong, Y., Hesse, D., Beutler, B., Manogue, K. R., Calvano, S., Wei, H., Cerami, A., and Shires, G. T. Cachectin/tumor necrosis factor induces lethal shock and stress hormone responses in the dog. *surg. gyn. obst.* 164:415-422, 1987b.

Tran Van Nhieu, J., Misset, B., Lebargy, F., Carlet, J., and Bernaudin, J. Expression of tumor necrosis factor- α gene in alveolar macrophages from patients with the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 147:1585-1589, 1993.

Trinchieri, G. and Perussia, B. Immune interferon: a pleiotropic lymphokine with multiple effects. *Immunol. Today* 6:131-136, 1985.

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., and Tada, K. Establishment and characterisation of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer* 26:171-176, 1980.

Tsuchiya, S., Kobayashi, Y., Goto, Y., Okumura, H., Nakae, S., Konno, T., and Tada, K. Induction of maturation in cultured human monocytic leukaemia cells by a phorbol diester. *Cancer Res.* 42:1530-1536, 1982.

Turner-Warwick, M., Burrows, B., and Johnson, A. Cryptogenic fibrosing alveolitis: clinical features and their influence on survival. *Thorax* 35:171-180, 1980.

Villanueva, P. O. F., Chikunguwo, S. M., Harris, T. S., and Stadecker, M. J. Role of IL-10 on antigen-presenting cell function for schistosomal egg-specific monoclonal T helper cell responses in vitro and in vivo. *J. Immunol.* 151:3192-3198, 1993.

Vincenti, M. P., Burrell, T. A., and Taffet, S. M. Regulation of NF- κ B activity in murine macrophages: effect of bacterial lipopolysaccharide and phorbol ester. *J. Cell Physiol.* 150:204-213, 1992.

Walters, E. H. and du Bois, R. M. Immunology and Management of Interstitial Lung Diseases. London: Chapman and Hall, 1995.

Wang, P., Wu, P., Siegel, M. I., Egan, R. W., and Billah, M. M. IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells. *J. Immunol.* 153:811-816, 1994.

Wanidworanun, C. and Strober, W. Predominant role of tumor necrosis factor- α in human monocyte IL-10 synthesis. *J. Immunol.* 151:6853-6861, 1993.

Watters, L. C., Schwartz, M. I., Cherniak, R. M., Waldron, J. A., Dunn, T. L., Stanford, R. E., and King, T. E. Idiopathic pulmonary fibrosis. Pretreatment bronchiolar lavage cellular constituents and their relationships to lung histopathology and clinical response to therapy. *Am. Rev. Respir. Dis.* 135:696-704, 1987.

Webster, N. R., Cohen, A. T., and Nunn, J. F. Adult respiratory distress syndrome - how many cases in the UK? *Anaesthesia* 43:923-926, 1988.

Willems, J., Joniau, M., Cinque, S., and van Damme, J. Human granulocyte chemotactic peptide (IL-8) as a specific neutrophil degranulator: comparison with other monokines. *Immunology* 67:540-542, 1989.

Wilson, A. G., de Vries, N., Pocoit, F., di Giovine, F. S., van der Putte, L. B. A., and Duff, G. W. An allelic polymorphism within the tumour necrosis factor α promoter region is strongly associated with HLA A1, B8 and DR3 alleles. *J. Exp. Med.* 177:557-604, 1993.

Windsor, A. C. J., Walsh, C. J., Mullen, P. G., Cook, D. J., Fisher, B. J., Blocher, C. R., Leeper-Woodford, S. K., Sugerman, H. J., and Fowler, A. A. Tumor necrosis factor- α blockade prevents neutrophil CD18 receptor upregulation and attenuates acute lung injury in porcine sepsis without inhibition of neutrophil oxygen radical generation. *J. Clin. Invest.* 91:1459-1468, 1993.

Wolpe, S. D. and Cerami, A. Macrophage inflammatory proteins 1 and 2: Members of a novel superfamily of cytokines. *FASEB J.* 3:2565-2573, 1989.

Xing, Z., Kirpalani, H., Torry, D., Jordana, M., and Gauldie, J. Polymorphonuclear leukocytes as a significant source of tumor necrosis factor- α in endotoxin-challenged lung tissue. *Am. J. Pathol.* 143:1009-1015, 1993.

Yamaguchi, E., Okazaki, N., Tsuneta, Y., Abe, S., Terai, T., and Kawakami, Y. Interleukins in pulmonary sarcoidosis. *Am. Rev. Respir. Dis.* 138:645-651, 1988.

Yanagawa, H., Sone, S., Haku, T., Mizuno, K., Yano, S., Ohmoto, Y., and Ogura, T. Contrasting effect of interleukin-13 on interleukin-1 receptor antagonist and proinflammatory cytokine production by human alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.* 12:71-76, 1995.

Yssel, H., de Waal Malefyt, R., Roncarolo, M., Abrams, J. S., Lahesmaa, R., Spits, H., and de Vries, J. E. IL-10 is produced by subsets of human CD4⁺ T cell clones and peripheral blood T cells. *J. Immunol.* 149(7):2378-2384, 1992.

Zhang, Y., Ramos, B. F., and Jakschik, B. A. Neutrophil recruitment by tumor necrosis factor from mast cells in immune complex peritonitis. *Science* 258:1957-1959, 1992.

Zhang, Y., Lee, T. C., Guillemin, B., Yu, M., and Rom, W. N. Enhanced IL-1 β and tumor necrosis factor- α release and messenger RNA expression in macrophages from idiopathic pulmonary fibrosis or after asbestos exposure. *J. Immunol.* 150:4188-4196, 1993.

APPENDIX

Lysis of Erythrocytes with Ammonium Chloride

Add 10 ml of 0.17 M tris buffer (20.6 g/l) to 90 ml of 0.16 M Ammonium Chloride solution (8.3 g/l) and incubate for 5 minutes at 37°C. Wash in complete media and resuspend.

Diff-Quick Staining (Baxter-Dade)

Immerse air-dried cytopins for 5 seconds in the fixative solution before 5 second immersion in solution 1, followed by 5 seconds in solution 2. Wash the slides in distilled water and examine under the microscope at x 400 magnification.

TNF- α ELISA BUFFERS

COATING BUFFER

Dissolve 4.27g BES (N, N-bis (2-hydroxyethyl)-2-amino ethane sulphonic acid - SIGMA B-9879) in 1 litre deionised water, adjust to pH 7 using 10% NaOH (BDH Analar). Store at ambient temperature for up to 1 week.

GLAZING BUFFER

Dissolve 10g lactose (BDH Analar) and 0.1g BSA (SIGMA) in 100 mls water on day of use.

BLOCKING BUFFER

1% BSA (SIGMA) in coating buffer on day of use.

PBS SOLUTION

Dissolve Dulbecco's formula (modified) without magnesium and calcium (Imperial labs) - 9.55g/litre deionised water. Store at ambient temperature for up to 2 weeks.

SAMPLE DILUENT

Add 200 mls heat inactivated normal mouse serum (Sigma) to 10 mls PBS containing 1% BSA.

CITRATE PHOSPHATE SUBSTRATE BUFFER

6.3 g citric acid monohydrate (Fluka) in 800 mls of deionised water. pH to 4.1 with KOH and adjust volume to 1 litre with deionised water. Stable for 6 months at 4°C.

TMB SUBSTRATE

Dissolve 120 mg tetramethylbenzidine substrate (Fluka) in 5 mls acetone. Make up to 50 mls with ethanol and add 500 µl of 30% H₂O₂. Store at an ambient temperature in the dark for up to 6 months.

IL-10 ELISA Buffers

COATING BUFFER

0.1M NaHCO₃ pH 8.2 in deionised water. Store at 4°C for up to 6 months.

PBS SOLUTION

80.0g NaCl, 11.6g Na₂HPO₄, 2.0g KH₂PO₄, 2.0g KCL (all Fisons) in 10 litres of deionised water, pH to 7.0. Store at ambient temperature for up to 2 weeks.

WASH BUFFER

0.5 ml Tween 20 (Sigma) added to 1 litre of PBS from above.

SUBSTRATE BUFFER

As above.

P-55 ELISA Buffers

COATING BUFFER

3.45 g NaH_2PO_4 and 4.45 g Na_2HPO_4 (Fisons) in 400 mls deionised water, pH 6.5. Store at an ambient temperature for 6 months.

PBS SOLUTION

80.0g NaCl , 11.6g Na_2HPO_4 , 2.0g KH_2PO_4 , 2.0g KCL (all Fisons) in 10 litres of deionised water, pH to 7.0. Store at ambient temperature for up to 2 weeks.

SAMPLE BUFFER

PBS solution plus 0.1% Tween 20 (Sigma) and 2% FCS

WASH BUFFER

0.5 ml Tween 20 (Sigma) added to 1 litre of PBS from above

TNF- α Immunostaining Buffers

PBS SOLUTION Dissolve 1 PBS tablet, without calcium and magnesium (Sigma) in 200 mls of deionised water. pH to 7.6 with NaOH . Store at ambient temperature for up to 2 weeks.

SUBSTRATE SOLUTION

Dissolve 1 tablet of 3,3'-diaminobenzidine (DAB) and 1 tablet of H_2O_2 (Sigma FAST™ DAB kit) in 1 ml of deionised water immediately before use.

Buffers used for Northern Analysis (all BDH-supplied unless stated)

DEPC-TREATMENT

Add 500 μl of DEPC (Sigma) to 500 mls of solution and incubate overnight at 37°C. Autoclave.

GUANIDINE ISOTHIOCYANATE (GITC) LYSIS BUFFER

4 M GITC (Fluka), 25 mM sodium citrate, 0.5% sarcosyl in sterile deionised water. Add 0.1 M 2-mercaptoethanol (Sigma) immediately prior to use. Store in the dark at 4°C for up to 4 weeks.

PHENOL EXTRACTION BUFFER

100 mM TRIS, 10 mM EDTA, 1% SDS (Sigma) made up in deionised water and autoclaved. Store at an ambient temperature for up to 1 month.

ETHIDIUM BROMIDE

Dissolve ethidium bromide (Sigma) in DEPC-treated water to 10 mg/ml. Store for up to 6 months at 4°C.

20X MOPS RUNNING BUFFER

0.4 M MOPS (Sigma), 0.02M EDTA, 0.2 M sodium acetate in DEPC-treated water. pH to 7.0 with solid NaOH and filter sterilise. Make up on day of use.

BROMOPHENOL BLUE SOLUTION

0.025g bromophenol blue and 3 mls of glycerol in 7 mls of DEPC-treated water. Store for up to 6 months at an ambient temperature.

AGAROSE GELS

3g of agarose (Sigma) in 230 mls of DEPC-treated water, microwaved for 4 minutes to dissolve. Add 15 mls of 20x MOPS buffer and 54 mls of 37% formaldehyde solution. Leave to cool slightly before pouring.

SAMPLE BUFFER

Add to each sample: 7 µl of 37% formaldehyde

5 µl of 20x MOPS buffer

1 µl ethidium bromide

20 µl formamide

20X SSC

3 M NaCl, 0.3 M sodium citrate in deionised water. Adjust to pH 7.0, DEPC-treat and autoclave. Store for up to 1 month at an ambient temperature.

HYBRIDISATION SOLUTION

5X SSC, 0.1% sarcosyl, 0.02% SDS and 1% Blocking Buffer (Boehringer Mannheim) in DEPC-treated water. Store for up to 1 month at 4°C.

BUFFER 1

0.1 M maleic acid, 0.15 M NaCl in deionised water. pH to 7.5 with NaOH, DEPC-treat and autoclave. Store for up to 1 month at an ambient temperature.

BLOCKING STOCK SOLUTION

10g blocking reagent (Boehringer Mannheim) in 100 mls of buffer 1. Microwave to dissolve and autoclave. Store for up to 6 months at 4°C.

WASHING BUFFER

0.3% Tween 20 in buffer 1, on day of use.

BUFFER 2

1:10 dilution of blocking stock solution in buffer 1. Store for up to 1 month at an ambient temperature.

BUFFER 3

0.1 M TRIS, 0.1 M NaCl, 50 mM MgCl₂ in deionised water. Adjust to pH 9.5 with HCL. Store in the dark for up to 6 months.

SODIUM SULPHITE

Make up an 18% solution in deionised water and store for up to 1 year in the dark at an ambient temperature.

PUBLICATIONS

Millar, A.B., Armstrong, L., van der Linden, J., Moat, N., Ekroth R., Westwick, J., Scallan, M., and Lincoln, C.J.R. Cytokine production and haemofiltration in children undergoing cardiopulmonary bypass. *Ann.Thorac.Surg.*, **56**:1499-1502 (1993).

Armstrong, L., Jordan, N., and Millar, A.B. Reduction of LPS-induced tumour necrosis factor- α release by macrophages/monocytes in response to interleukin-10. *Am.Rev.Respir.Dis.*, **149**:A1066 (1994).

Armstrong, L., and Millar, A.B. Differences in tumour necrosis factor expression and release by three distinct alveolar macrophage populations. *Eur.Respir.J.* **7**:247S (1994)

Armstrong, L., and Millar, A.B. Tumour necrosis factor- α regulation by interleukin-10 in alveolar macrophages from patients with sarcoidosis or fibrosing alveolitis. *Thorax* **50**:457P (1995).

Armstrong, L., and Millar, A.B. Interleukin-10 detected in the plasma and bronchoalveolar lavage of patients with the adult respiratory distress syndrome. *Am.Rev.Respir.Dis.* **151**:A76 (1995).

Armstrong, L., and Millar, A.B. Suppression of LPS-induced tumour necrosis factor mRNA in alveolar macrophages and peripheral blood monocytes in response to interleukin-10. *Am.Rev.Respir.Dis.* **151**:A829 (1995).

Armstrong, L., Jordan, N., and Millar, A.B. IL-10 regulation of TNF- α from human alveolar macrophages and peripheral blood monocytes. *Thorax* **51**: 143-149 (1996).